

CANCER RESEARCH

Vol. 9

JANUARY 1949

No. 1

CONTENTS

Announcement	Charles Huggins	1
Editorial Foreword	Paul E. Steiner	2
Paul G. Zamernik and Mary L. Stephenson, A Comparison of Activators of Proteolytic Enzymes and Peptidase in Normal Rat Liver and Hepatoma	5	
Henry W. Scherp and Jerome T. Syvertson, A Chemical Investigation of Keratin and Collagen Derivatives from Rabbit Papillomas (fibroepithelioma)	12	
David Moritz, Otto Saphir, and Alfred Strauss, Effect of an Antitumoral Cytotoxic Serum on the Brown-Pearce Carcinoma of the Rabbit	17	
Logan O. Jones, In vitro Studies on the Effect of Spleen, Striated Muscle, and Kidney upon the Growth of Sarcoma 180 and Mammary Carcinoma of Mice	27	
Min-Hsin Li and W. U. Gardner, Further Studies on the Pathogenesis of Ovarian Tumors in Mice	35	
Sr. M. Agatha Richl and Sr. M. Petra Lenta, Dihydrogenate Studies of Tissue from Normal and Tumor-bearing Mice. I. Total Dihydrogenate Activity	42	
Sr. M. Petra Lenta and Sr. M. Agatha Richl, Dihydrogenate Studies of Tissue from Normal and Tumor-bearing Mice. II. Lactic and Malic Dihydrogenases	47	
Hans Schlumberger and Baldwin Lucké, Solid Intracocular Transplantation of Free Carcinoma for Fourteen Generations	52	
Doris H. Bender, Charles E. Friedgood, and Henry F. Lee, Transplantation of Heterologous Tumor by the Intravenous Inoculation of the Chick Embryo	61	

THE OFFICIAL ORGAN OF THE
AMERICAN ASSOCIATION FOR CANCER RESEARCH
Published by THE UNIVERSITY OF CHICAGO PRESS

CANCER RESEARCH

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Cambridge University Press, Bentley House, 200 Euston Road, London, N.W. 1, England. Prices of yearly subscriptions and of single copies may be had on application.

Business communications, remittances (in United States currency or its equivalent), and subscription should be addressed to THE UNIVERSITY OF CHICAGO PRESS, 5750 Ellis Avenue, Chicago 37, Illinois. All other communications should be addressed to Paul E. Steiner, M.D., University of Chicago, Chicago 37, Illinois.

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Entered as second-class matter, December 16, 1946, at the post office at New Haven, Conn., under the Act of March 3, 1879. Application pending transfer to Chicago, Ill.

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VOL. 9

FEBRUARY 1949

No. 2

CONTENTS

Howard A. Bern. Some Effects of Long-continued Estrogen Treatment on Male Dutch Rabbits	65
Robert A. Kitzler, Barbara Mulliken, and Joseph C. Turner. Histological Appearance of Monkey Sarcoma 180 Infected by Vaccinia Virus	71
R. M. Mulligan. Primary Liver-Cell Carcinoma (Hepatoma) in the Dog	76
A. C. Griffin, G. C. Clayton, and C. A. Baumann. The Effect of Certain and Methoxyquin on the Retention of Hepatic Riboflavin and on the Development of Liver Tumors in Rats Fed Certain Azo Dyes	81
James B. Murphy and Ernest Sturm. The Effect of Dieldrin-Residues on the Incidence of Leukemia in Male Mice of the Rockefeller Institute Leukemia Series (R.I.L.)	88
Frances L. Haven, Chelliss Randall, and W. R. Bloom. The Citric Acid Content of Tumor Tissue and of Tumor-bearing Rats	93
Arthur Kirschbaum. Induction of Mammary Cancer with Methylcholanthrene. II. Histological Similarity between Carcinogen-induced Tumors and Certain Mammary Neoplasms Occurring Spontaneously	95
J. M. Price, J. A. Miller, F. C. Miller, and G. M. Weber. Studies on the Initiation-Delay Carcinogenesis of Liver and Liver Tumor from Rats Fed 4-Dimethylaminobutene	97
George T. Lewis, Walter L. Bloom, and Charles W. Smart. Studies of the Rescondensation of Nucleic Acids with a Tissue Polypeptide	107
John B. Storer and Clarence C. LaSallebaugh. The Effects of Hepatic and Splenic Extracts from Human Lymphomata upon the Lymphatic System of Experimental Animals	108
Salter Marx. The Influence of Normal and Cancer Blood on Tyrosinase Activity <i>in vitro</i>	113
Elizabeth Lowenthal, Splenic Lymphomatous in Rats Bearing Intraperitoneal Implants of Butter Yellow	121
New Books	127
Announcement of Annual Meeting	128

THE OFFICIAL ORGAN OF THE

AMERICAN ASSOCIATION FOR CANCER RESEARCH

Published by THE UNIVERSITY OF CHICAGO PRESS

Fortieth Annual Association Meeting, Detroit, April 16 and 17, 1949



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Entered as second-class matter, December 16, 1946, at the post office at New Haven, Conn., under the Act of March 3, 1879. Application pending transfer to Chicago, Ill.

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CANCER RESEARCH

Vol. 9

OCTOBER 1949

No. 10

CONTENTS

C. C. Clayton and G. A. Baumann. Diet and Azo Dye Tumors. Effect of Diet During a Period When the Dye is Not Fed	355
A. Rosin. Early Changes in the Lungs of Rats Treated with Urithione (Ketyl Carbamate)	365
Hugh J. Creech and Reed F. Hankwitz. Further Studies of the Immunological Properties of Polysaccharides from <i>Serratia marcescens</i> (Bacillus prodigiosus) III. Passive Immunization Against the Lethal Activity of the Polysaccharides with Fractions of Monoclonal Antisera Prepared by a Single Injection of Polysaccharide	375
Scientific Proceedings, 1949, American Association for Cancer Research, Inc.	385
Business Proceedings, 1949, American Association for Cancer Research, Inc.	405

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CANCER RESEARCH

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NOVEMBER 1949

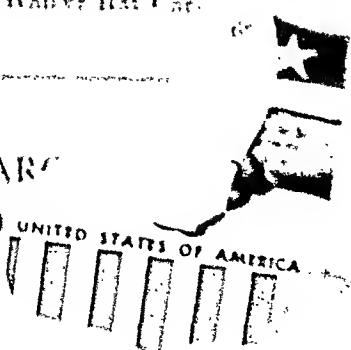
No. II

CONTENTS

Carl S. Vestling, Jesse N. Williams, Jr., Seymour Kaufman, Richard E. Maxwell, and Henry Quastler. The Oxidation of Octanoate by Liver Homogenates from Normal Mice	613
Eugene Roberts and Sam Frankel. Free Androstanediol in Normal and Neoplastic Tissues of Mice as Studied by Paper Chromatography	615
Dale Rex Coman, Richard H. Eisenberg, and Morton McCutcheon. Factors Affecting the Distribution of Tumor Metastases. Experiments with V ₁ Cells and <i>in Vitro</i> Cultures	619
J. A. Miller, R. W. Sapp, and E. C. Miller. Carcinogenic Action of Certain H_2^3 -labeled Derivatives of 4-Dimethylaminostilbene in the Rat	633
George H. L. Dillard, H. Rowland Postall, and Alfred Chamutin. Electrolytic Nitrogen, Lipide and Enzyme Studies of the Plasma and Plasma Fractions in Cancer	637
George H. L. Dillard and Alfred Chamutin. The Proteins and Amino-acids of Tissues of Patients with Cancer and Other Diseases	645
Kristen Arnesen, Yvette Goldsmith, and Anna Dean Dulacry. Antibodies to proteins which Segregated from Spleens of Normal and Leukemic Mice	651
Harold M. Rabinovitz. A Correlation of Histopathology of Human Tissue with Benign or Early and Growth	659
Henry A. Sloviter. The Distribution and Action of a Redoxitive Dye in Tumors of Mice	667
William H. Fishman, A. Wayne, and E. Hamburger. The Evaluation of Diagnostic Tests for Cancer, II. Inhibition of Serum Alkaline Phosphatase by Zinc Iodide (Zn-I ₂) Test	671
Herbert Silverstone and Albert Tannenbaum. Influence of Thyroid Hormone on the Formation of Induced Skin Tumors in Mice	673
R. M. Ballantyne and E. W. McHenry. Vitamin B ₆ and Biotin in Human Cancer Tissue	683
John W. Green, Jr., and G. C. Lushbaugh. Histopathologic Study of the Mode of Inhibition of Cellular Proliferation: Effect of 4-Dimethylaminostilbene on the Growth of Walker Rat Carcinoma 256	687

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JANUARY 1949

Number 1

Announcement

IT IS a pleasure to announce that with the issue *CANCER RESEARCH* is appearing under the auspices of a new Editorial Board and with a new Publisher. Owing to the vast and independent difficulties of nearly all kinds, there have been great delays in the publication of this journal which we hope have now ended.

CANCER RESEARCH is the official organ of publication of the American Association for Cancer Research, Inc. At the annual meeting of the Association in Atlantic City in 1946, the Board of Directors authorized the formation of a holding company for *CANCER RESEARCH*, the officers of which were to be elected with the officers of the Association. This has been established, and the members now own and publish all to the new corporation, Cancer Research, Inc. The Board of Directors of the Association has considered it essential to fulfil two obligations:

1. To give prompt and wide dissemination of progress research in the field of cancer.
2. In attempt to reduce the present difficulties of publication, *CANCER RESEARCH* has been organized.

The officers of Cancer Research, Inc., as well as the very well distinguished Board of Editors and the Publisher, will do all they can in order to accomplish these ends. They depend at the same time upon the enthusiastic cooperation of the members of the American Association for Cancer Research, Inc., our sponsors, and of the research workers on problems of neoplastic growth that *CANCER RESEARCH* has occupied in the past.

Charles Huggins, President

Editorial Foreword

WITH the advent of a new editorial staff, it is customary to make a statement regarding editorial policy. *CANCER RESEARCH* is fortunate in that first its Editorial Committee and later its editors, Dr. William H. Woglom, Dr. S. Bayne-Jones, and Dr. Baldwin Lucké, with their staffs, have set high standards. It is our present policy to emulate them, but the general editorial policy will remain unchanged. Dr. Lucké, who has had to relinquish the position of editor-in-chief because of press of other duties, has generously consented to remain as an editor, giving to the new editorial staff the benefits of his experience.

CANCER RESEARCH is devoted to reporting the results of fundamental investigations directed toward the understanding and ultimate conquest of cancer. Original reports of laboratory and clinical research, chiefly experimental, will be published regardless of whether the subjects are mice or men. Comprehensive but concise general reviews of subjects selected by the editorial staff will be published from time to time. Short papers, even preliminary, if so marked, will be welcome. News items, announcements, comments, and death notices will also be published. Other sections will be added as warranted. It is hoped that such prompt publication can be offered to papers of wide and active interest that a copy of *CANCER RESEARCH* will be a necessity on the desk of every scientist and physician concerned with cancer.

With the great expansion in cancer research now under way and the limitation of printing space dictated by budgetary considerations, it will be impossible to print all papers submitted to the editors. Critical selection between them will be necessary, and some authors will, of necessity, be disappointed. Past policy of careful editorial selection by specialists in each field will be rigidly followed. The role of the editors and advisory editors will be to help in the selections, but the final responsibility must rest with the editor-in-chief, who alone will have access to all editorial opinions and be able to judge the apparent comparative merit of the papers submitted. Knowing that, regardless of how carefully the selections are made, some persons will disagree with the choices, the editor-in-chief is fully reconciled to receiving adverse criticism and to loss of all of his friends if this becomes necessary in order to maintain what he regards as the highest standards for *CANCER RESEARCH*. It is expected that *CANCER RESEARCH* can eventually be enlarged.

It is hoped that in these pages, among other benefits, investigators will find an adequate and prompt publication outlet for their results, readers a fertile and reliable source of information, physicians a flood of ideas and inspiration which they will use in the clinic, and the sponsors satisfaction in having had a vital part in an important job well done.

PAUL E. STEINER

usually showed evidence of diffuse, nodular cirrhosis of a mild degree but was grossly nonmalignant. Thus in a single animal there were present both malignant and nonmalignant areas of liver, clearly separated from each other (Fig. 1). We have found that, under similar conditions, rats left on Kline's diet (21) for 6 months or more develop livers studded and infiltrated with hepatomas, with little tissue remaining that can be termed nonmalignant or normal. On the other hand, if the dye is discontinued at the end of the fourth month, a relatively few hepatomas arise in isolated areas of a single liver. We have, therefore,

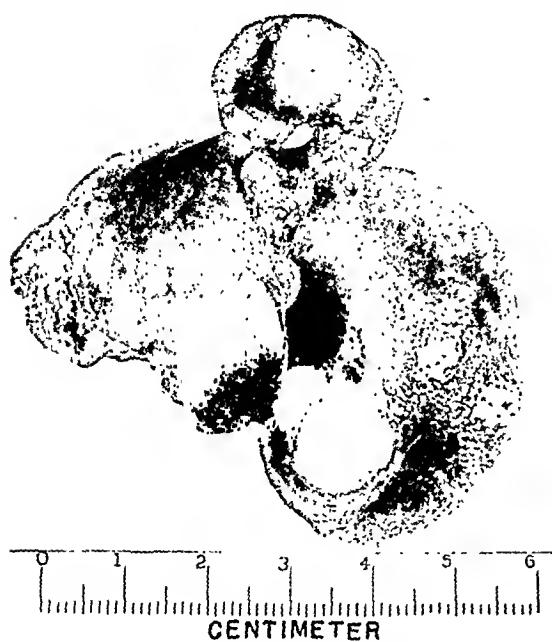


FIG. 1.—Typical liver, showing hepatoma nodules

taken advantage of this circumstance in preparing tumors for the present experiment.

The opaque, white hepatoma nodules were carefully dissected free from surrounding grossly nonmalignant tissue. Hepatoma nodules obtained from a single liver varied in size from a few millimeters to 1 to 1.5 cm. In the case of the larger nodules, particular care was taken to select only the peripheral, grossly non-necrotic, firm portions. Sections were also taken from such large nodules for histological verification. Thus a quantity of hepatoma nodules weighing from 3 to 10 gm., usually the former, was obtained from a single liver. In a number of experiments an equal weight of tissue was likewise taken from the grossly nonmalignant parts of these livers, and histological sections were saved from these regions. At the

same time, a control liver was frequently obtained and prepared as described above.

Ultrafiltration.—A 1:1 homogenate of liver or hepatoma was quickly made with distilled water, using a Potter-Elvehjem apparatus (24). The homogenized livers were then placed individually in small Cellophane bags³ inside side-armed Erlenmeyer flasks in the cold-room at 3° C. and were allowed to filter under the influence of a negative

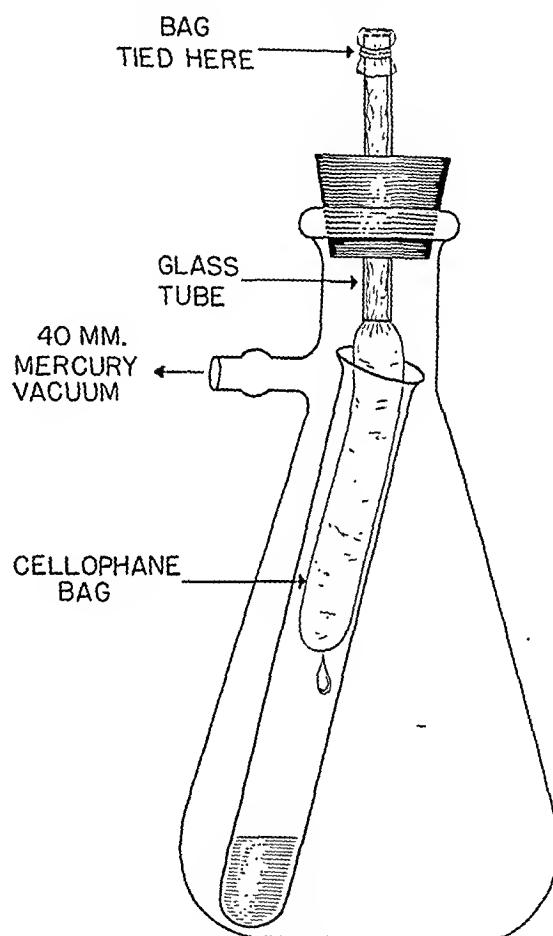


FIG. 2.—Ultrafiltration apparatus

pressure of approximately 40 mm. of mercury (Fig. 2). This filtration pressure was selected so as to be just under the breaking-point of the bags. Ultrafiltration was chosen as a method for separating the substances of large molecular weight present in liver tissue from compounds of smaller molecular weight because it seemed to introduce less possibility of artifacts than did acid precipitation methods. One to 3 cc. of water-clear, protein-

³ Visking Cellulose Sausage Casings, 14/32-inch "No Jax" Casing.

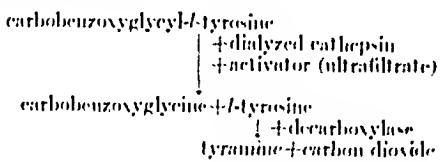
free ultrafiltrate were collected during the following 4 to 6 hours. It was found that, if the ultrafiltrate was allowed to collect overnight, the activating ability of an aliquot was less potent than that of one obtained during the early hours. Storage of ultrafiltrates over a period of days on dry ice, however, resulted in no appreciable loss in their ability to activate the catheptic enzyme.

ENZYMATIC DETAILS

1. Preparation of cathepsin.—Several separate preparations of cathepsins were made by ammonium sulfate fractionation of fresh hog kidneys, according to the procedure of Fruton and Bergmann (12). In order to remove, as far as possible, all traces of natural activators, the final products were dialyzed at 3° C. for 9 to 13 days, first against several changes of copper-distilled water and, finally, against glass-distilled water.

The activity of the cathepsin preparations was tested toward the substrate carbobenzoxylglycyl-*L*-tyrosine, by means of a manometric method (30), both in the presence and in the absence of added activator. Each preparation of cathepsin was divided into 10-cc. portions. These were frozen in Eustroid tubes in a dry-ice box, were thawed out individually, and used immediately. The frozen enzyme solution retained its activity, but the activity decayed over a period of hours after thawing.

2. Determination of catheptic carboxypeptidase.—Although such a preparation as that mentioned above contains a number of catheptic enzymes, it was convenient to study the effect of activators in detail on only two enzymes—the carboxypeptidase which hydrolyzes the substrate carbobenzoxylglycyl-*L*-tyrosine, and the trypsinase, or more strictly benzoylargininamidase (17), which hydrolyzes benzoyl-*L*-argininamide. The catheptic carboxypeptidase method depends on the ability of a tyrosine decarboxylase (obtained from *Streptococcus faecalis*) to liberate carbon dioxide from free *L*-tyrosine, but not from the peptide carbobenzoxylglycyl-*L*-tyrosine. The reaction sequence involved is outlined below:



Reactions were carried out in Wurzburg-type constant-volume respirometers, at 25° C. The gas phase was nitrogen, purified of traces of oxygen by passage over hot copper. In agreement with previous work (19), we have found higher catheptic activity when the reactions have been run in the absence of oxygen. Unless otherwise stated, the composition of the test solution was as follows:

Five-tenths cubic centimeters of dialyzed cathepsin, 0.3 cc. of 0.055 M carbobenzoxylglycyl-*L*-tyrosine,⁴ and 0.6 cc. of decarboxylase made up in 0.4 M citrate buffer. The pH was 5.4 to 5.6 in various experiments, but in a single experiment there was less than 0.1 pH units difference among the various flasks. The decarboxylase was prepared as previously described (30).

Provided that the decarboxylase is present in excess, the rate of carbon dioxide evolution serves as a measure of the rate of the cathepsin reaction (30). During the early portion of the hydrolysis, the reaction closely follows zero-order kinetics. Likewise, if the dialyzed cathepsin is completely inactive in the absence of added activator, the rate of carbon dioxide evolution reflects the amount of cathepsin activation provided by the ultrafiltrate. Even in the absence of added activator, however, the dialyzed enzyme was able to split this substrate slightly. This suggests that all the natural activator may not have been dissociated from the enzyme during the prolonged dialysis. The relationship between concentration of glutathione added to the test solution and rate of peptide hydrolysis (as reflected by carbon dioxide evolution) is illustrated in Fig. 3.

It may be seen from Fig. 3 that the activity of the dialyzed cathepsin toward the substrate carbobenzoxylglycyl-*L*-tyrosine is a function of the glutathione concentration added to the test solution.

3. Determination of catheptic trypsinase (benzoylargininamidase).—A smaller number of experiments was performed, in which the action of dialyzed cathepsin preparation on the substrate benzoyl-*L*-argininamide⁵ was tested at pH 5 both in the presence and in the absence of added activator. The experimental details were as follows: Reactions were run in 2.5-cc. glass-stoppered volumetric flasks, incubated in a water bath at 37° C. in the presence of air. The flasks contained 1.0 cc. of 0.1 M benzoyl-*L*-argininamide, 0.2 cc. of 1 M citrate buffer at pH 4.72, 0.4 cc. of cathepsin, and 0.4 cc. of ultrafiltrate. With each experiment one control was run in which 0.4 cc. of distilled water was used in place of ultrafiltrate, and another control in which 0.4 cc. of 0.05 M cysteine was simi-

⁴The authors are indebted to Dr. Max Bremer and the A. G. Geigy Co., Basle, Switzerland, for generous supplies of carbobenzoxylglycyl-*L*-tryptophane and of *L*-leucinamide.

⁵Obtained through the courtesy of Dr. M. S. Dunn and the Amino Acid Manufacturers, Los Angeles, California.

larly used. Autolysis controls were also included in which water was used in place of the substrate. There was no autolysis at the end of an hour. Duplicate aliquots were removed at zero time, and the reactions were all terminated at 60 minutes.

The pH's of aliquots from all flasks were checked at the beginning and end of each experiment by means of a micro glass electrode assembly. Aliquots of 0.2 cc. were diluted to 1.2 cc. with distilled water and were tested. With rare exceptions, the pH's at this dilution were between 5.40 and 5.50 throughout the experiments.

The flasks were shaken briefly by inversion at 15-minute intervals during the course of the reaction. Individual flasks were removed from the warm bath at 60 minutes, placed in ice water, and three 0.2-cc. aliquots were removed. The aliquots were pipetted into aeration tubes containing 5 drops of caprylic alcohol, 2 drops of castor oil,

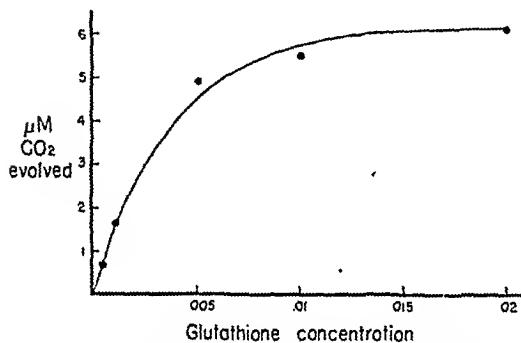


FIG. 3.—Relationship of cathepsin activity to glutathione concentration. The flask contents were as follows: 0.5 cc. of 13-day dialyzed cathepsin; 0.3 cc. of 0.055 M carbobenzoxy-glycyl-l-tyrosine; 0.6 cc. of decarboxylase (20 mg. of dried powder per cubic centimeter of 0.4 M citrate buffer, pH 5.3); and 0.5 cc. of glutathione solution. The pH's of the various flasks, as determined at the end of 1 hour's reaction, ranged from 5.25 to 5.43. The temperature was 27° C., and the gas phase was purified nitrogen. A control flask was run in the absence of glutathione, and the value obtained (0.7 μ moles of CO₂ liberated per hour) was subtracted from the other readings. Glutathione concentration is expressed in moles per liter, and carbon dioxide in μ moles evolved per hour.

0.5 cc. of a half-saturated solution of potassium carbonate, and 0.8 cc. of distilled water. The tubes were stoppered, and aeration was begun immediately. The ammonia was collected in boric acid and was titrated (27), using 0.0782 N sulfuric acid and a Rehbung burette. The average of the replicate titrations was used in each case. In following the splitting-off of the amide group by the cathepsin, ammonia aeration, according to a modification of the method of Sobel, Meyer, and Gottfried (27), was found to be more convenient and accurate in our hands than was the more classical titration procedure of Grassmann and Heyde (15).

More effort was expended on studies on carbobenzoxyglycyl-l-tyrosine, however, than on those with benzoyl-l-argininamide, because of the greater precision and ease of manipulation of the manometric method used in the study of the enzymatic hydrolysis of the former substrate.

4. *Determination of l-leucinamidase.*—This enzyme was used to provide an example of the behavior of the group of metal-activated peptidases whose pH optimum is in the vicinity of pH 8: A crude preparation of peptidase was made from rat liver in the following way: 9 gm. of normal rat liver were minced and then homogenized with 27 cc. of water. The homogenate was twice filtered through a Buchner funnel with the aid of Hy-Flo Supercel. To the filtrate was added an equal volume of ice-cold acetone. The precipitate was washed 3 times with ice-cold acetone. To 300 mg. of dried precipitate, 18 cc. of water were added. The extract was centrifuged, and 17 cc. of a clear, reddish supernatant solution were obtained. This solution contained an enzyme which actively hydrolyzed l-leucinamide at pH 8.

The experimental details were as follows: flasks contained 1.0 cc. of 0.1 M l-leucinamide (previously adjusted to pH 8); 0.2 cc. of 0.4 M borate buffer at pH 8; 0.4 cc. of a 1.5 aqueous dilution of enzyme solution; 0.1 cc. of 0.02 M manganese chloride (when used); 0.4 cc. of ultrafiltrate (when used); and distilled water to 2.0 cc. The reaction temperature was 37° C. At 15-, 30-, and 45-minute intervals, 0.2-cc. aliquots were removed and were titrated according to the technic of Grassmann and Heyde (15).

5. *Determination of reduced glutathione.*—The most specific procedure for the determination of tissue glutathione concentration is the manometric method of Woodward (29). This method depends on the ability of reduced glutathione to serve as a coenzyme for the yeast enzyme, glyoxalase, in catalyzing the conversion of methylglyoxal to lactic acid. The lactic acid formed during the reaction displaces carbon dioxide from a carbon dioxide-bicarbonate buffered system, and the carbon dioxide evolution is measured manometrically. Several batches of yeast were tested for glyoxalase activity, and calibration curves were set up, in which varying amounts of glutathione were added to the glyoxalase. It was found impossible to free the yeast glyoxalase completely from its naturally accompanying coenzyme. Even without the addition of glutathione, therefore, there was considerable glyoxalase activity. Results have been corrected for this blank value in each experiment by running flasks without added glutathione.

RESULTS

A. ACTIVATING EFFECT OF ULTRAFILTRATES ON DIALYZED CATEPSIN

1. *Results on catheptic carboxypeptidase.*—In Table 1 is tabulated the effect of ultrafiltrates on the activity of dialyzed cathepsin, using the substrate carbohenoxyglycyl-L-tyrosine. It will be noted that there is some enzymatic activity present even when no ultrafiltrate is added to activate the cathepsin. Apparently, not all the natural cathepsin activator is removed by dialysis. The average of the values in the first column of Table 1 may thus be subtracted from the averages of values in the other columns to obtain figures for

TABLE 2

EFFECT OF ULTRAFILTRATES ON THE ACTIVITY OF A DIALYZED CATEHEPTIC ENZYME USING CARBOHENOXYGLYCYL-TYROSINE AS SUBSTRATE*

No. ultrafiltrate	Control ultrafiltrate	Regenerating liver	Fetal liver	Hepatoma
6.7	17.1	28.0	46.8	8.3
5.0	26.0	26.9	26.6	17.5
6.5	26.6	10.6	33.2	11.1
	17.3	29.2	15.5	7.0
	16.1	29.2	16.8	8.1
	20.6	18.6	12.1	
			23.0	
	17.4			
	26.4			
6.1	21.0†	23.7‡	39.8‡	13.1

* Experimental details same as those given in Table 1.

† Difference from hepatoma ultrafiltrate probably significant ($p < 0.05$).

‡ Difference from hepatoma ultrafiltrate significant ($p < 0.01$).

the amount of activation provided by the addition of the three types of ultrafiltrates. The results indicate less activating ability in the ultrafiltrates of hepatoma nodules than in the ultrafiltrates from the nonmalignant parts of the same livers. There is likewise less activating ability in the hepatoma ultrafiltrates of livers of control rats.

In Table 2 are listed the results of a completely separate series of experiments in which two additional types of control livers were used, namely, regenerating and fetal livers. The regenerating livers were produced by performing partial hepatectomy (5), with removal of the regenerating liver 24 hours later. Each figure in the fetal liver column represents an ultrafiltrate obtained from the pooled livers of the fetuses of a single mother. As before, hepatoma nodules were carefully dissected out of individual livers. Another preparation of cathepsin was used for these experiments. Since the order of activity of this enzyme preparation, per milligram of protein nitrogen, was considerably less than that of the previous preparation; and, because the amount of natural activator dialyzed out might also be different, this series of

experiments must be viewed as a unit separate from those in Table 1.

The results again show a lower activation effect of the hepatoma nodule ultrafiltrates than that found in the controls. It is noteworthy that the activating ability of the fetal ultrafiltrates is particularly high.

2. *Results on benzoylargininamidase (trypsinase).*

—The effect of ultrafiltrates on benzoylargininamidase activity is summarized in Table 3. It will be noted that the dialyzed enzyme has little activity in the absence of added activator. The addition of 0.01 M cysteine or glutathione provides maximal activation. Although there is overlapping of values in the two series, the average concentration of benzoylargininamidase activator is less in the hepatoma than in the control ultrafiltrates. The results thus fall in line with those on the activation of catheptic carboxypeptidase.

TABLE 3

EFFECT OF ULTRAFILTRATES ON BENZOYLARGININAMIDASE ACTIVITY*

No. activator added	0.01 M Cysteine added	Control ultra- filtrate $\times 10^3$	Hepatoma ultra- filtrate $\times 10^3$
1.9	62	9.8	2.6
1.2	59	9.9	9.6
2.7	55	9.4	3.4
2.6	62	5.6	1.6
1.6	63	6.3	1.8
1.3	61	9.3	10.8
2.0	58	9.7	8.5
1.6	58	10.4	2.4
1.5	59	7.8	1.5
0.14	10	7.9	2.0
1.21	42	10.1	3.4
1.14	45	6.8	5.7
1.6	55	8.4	1.51

* Each horizontal line represents an experiment carried out on a single day. Each figure in cols. 3 and 4 represents a separate liver. Results are expressed as 10^{-3} mole split per hour per mg. ultracentrifuged filtrate. One hundred per cent hydrolysis of substrate on this basis would represent 100×10^{-3} mole.

† A second preparation of enzyme was used for the last 3 experiments. The maximal activity per milligram protein nitrogen was somewhat lower than that of the previous preparation. A correction could conveniently be applied in order to bring the whole series of figures for the last 3 experiments more closely in line with the previous ones. This was not, however, done.

‡ Difference from control ultrafiltrate significant ($p < 0.01$).

3. *Results on L-leucinamidase.*—The enzyme used in these few experiments was prepared from rat liver as described above. When manganese chloride was added to the test solution, the enzyme hydrolyzed L-leucinamide very actively (50 per cent of the substrate was hydrolyzed in 45 minutes). Without the addition of manganese, however, there was only slight activity. The addition of ultrafiltrates of a normal liver and of a hepatoma produced no activating effect. This en-

zymic is, of course, quite different from the cathepsins discussed above. The results thus indicate that no appreciable amount of activator for this enzyme has been found in ultrafiltrates of either normal or malignant liver by this technic.

B. STUDIES ON CHEMICAL NATURE OF CATEPSIN ACTIVATORS PRESENT IN ULTRAFILTRATES

1. *Nitroprusside test.*—Since it has been known (13) that certain compounds containing sulphydryl groups may serve as cathepsin activators, frozen aliquots of the ultrafiltrates were thawed and tested for their total sulphydryl activity by means of the nitroprusside reaction (18). The colorimet-

TABLE 4

SODIUM NITROPRUSSIDE TEST OF SULPHYDRYL GROUP CONCENTRATIONS IN ULTRAFILTRATES OF VARIOUS TYPES OF RAT LIVERS*

Control	Hepatoma	TYPE OF LIVER		
		Control-hepatoma†	Fetal	Regenerating
60	0-5	60	20	100
60	5	80	15	60
50	0-5		5	80
60	0-5		5	
60	0-5		15	
80	0-5			
60				

* Each figure represents a determination on a single liver. An arbitrary color standard was set at 100, and the figures have meaning only in relation to one another.

† This term signifies the grossly nonmalignant portion of a liver containing a hepatoma.

ric results could be roughly quantitated and are summarized in Table 4. The figures suggest that the sulphydryl concentration of the hepatoma is less than that in the normal livers, in regenerating liver, and in the nonmalignant part of the hepatoma-containing livers. The sulphydryl concentration of the fetal livers was, however, only slightly higher than that of the hepatomas. It thus appeared desirable to characterize more closely the nature of the sulphydryl-containing compounds responsible for the nitroprusside test.

2. *Filter-paper chromatograms.*—Such chromatograms of ultrafiltrates of normal livers, prepared according to Consden, Gordon, and Martin (7), indicated the presence of glutathione in considerable concentrations and a relative paucity of cysteine.

3. *Glyoxalase method.*—The glyoxalase method of Woodward (29)⁶ was then used to quantitate the concentration of reduced glutathione. The results of these studies are recorded in Table 5.

The points of interest are the low glutathione concentrations of the hepatoma nodules and of the

⁶ The dihydroxyacetone from which methylglyoxal was prepared was kindly furnished by Drs. Fritz Lipmann and C. B. Anfinsen.

fetal livers. Thus the low cathepsin-activating ability of the hepatomas appears to fit well with their low, reduced glutathione concentrations. This explanation cannot, however, hold when applied to the fetal liver ultrafiltrates. In the latter case there is a high cathepsin-activating ability but a low reduced glutathione concentration.

The possibility was considered that the glutathione might be present in oxidized form in the hepatoma and thus not be detectable by the glyoxalase method, which measures only reduced glutathione. To test this possibility, the electrolytic reduction method of Dohan and Woodward (8) was set up. By this method glutathione in the oxidized form is reduced and then tested as before by the glyoxalase method. It was found that very little oxidized glutathione was present in the hepatomas tested, and that this, therefore, could not be the explanation for the low concentrations of glutathione found in the hepatomas.

TABLE 5
GLUTATHIONE CONCENTRATION OF VARIOUS TYPES OF ULTRAFILTRATES*

Control liver	Regenerating liver	Fetal liver	Control-hepatoma†	Hepatoma
0.093	0.117	0.013	0.079	0.018
.078	.108	.020	.112	.000
.116	.058	.018	.071	.005
.125	0.050	.033	.093	.003
.091		0.018	.117	.011
.080			0.068	.009
.071				.009
.118				.013
0.085				.020
				.009
				.003
				.000
				.006
				.003
0.095	0.083	0.020	0.090	0.010

* Figures indicate milligrams glutathione per 0.1 cc. of ultrafiltrate.

† Nonmalignant part of hepatoma-containing liver.

4. *Sulfosalicylic acid filtrates.*—In order to check on the possibility that ultrafiltration through the Cellophane bag might introduce some unexpected artifact with respect to the glutathione concentrations of the various groups of livers, a series of sulfosalicylic acid filtrates of control livers and hepatomas were run in parallel on the same livers from which the ultrafiltrates were prepared. The glutathione concentrations were in satisfactory agreement by use of the two independent methods.

DISCUSSION

In order to bring the result into focus with current conceptions (26, 25) of protein metabolism within a living cell the following diagrams are pre-

sented (cf. Fig. 4). In a normal adult cell (*N*) in nitrogen equilibrium (*1.*), protein is being synthesized at a rate (*A*) similar to that (*B*) at which it is being degraded. There is thus no net change in the intracellular protein concentration. In a malignant cell (*M*), protein may either be synthesized at an accelerated rate (*2.*), degraded at a decreased rate (*3.*), or both aspects of the process may be affected (*4.*).

There are at least 3 families of enzymes which appear to be concerned in protein and peptide degradation within cells: (*a*) the cathepsins, active at weakly acid pH's; (*b*) the peptidases, active at neutral and slightly alkaline pH's; and (*c*) the more obscure dehydropeptidases. The present experiments suggest that the concentration of cathepsin activators may be decreased in the hepatomas. Thus the present results are consonant with either situation (*3.*) or (*4.*).

We have recently (32) found C^{14} -labeled alanine to be incorporated into the proteins of primary rat hepatoma slices more rapidly than into the proteins of normal liver slices. These data imply an increase in the rate of protein synthesis in the hepatoma *in vitro* but give no information on the rate of protein degradation. In summary, these two fragmentary pieces of evidence, therefore, suggest that situation (*4.*) (Fig. 4) may be the correct one for this type of hepatoma. It is, of course, possible that either situation (*2.*) or situation (*3.*) (Fig. 4) may apply for other tumors.

The question as to whether the catheptic enzymes play a role in protein *synthesis* within the cell, as well as in protein degradation, has, of course, not been settled. Recent work from several laboratories (4, 6, 10, 23), however, favors the point of view that a different set of enzymes is involved in protein synthesis from those concerned with protein degradation.

The data on fetal livers require special comment. Since the glutathione concentration is low but the cathepsin activator concentration high in the ultrafiltrates of fetal liver, there must be some substance other than glutathione responsible for this high order of activation. The nature of such a hypothetical substance is unknown.

It has been pointed out recently (22) that the glutathione concentration of rat livers depends on the diet fed. In the present experiments, animals in all groups except fetal had been on the same control diet for 2 months, prior to sacrificing, which minimizes the possibility that variations in diet might have affected the glutathione concentrations of the several groups.

The glutathione concentration of normal and malignant tissues has been the subject of consider-

able study, as pointed out in a recent review (28). Fewer comparisons, however, have been made between glutathione concentrations of malignant tissues and their homologous normal counterparts (14, 16, 20). The present findings are in qualitative agreement with those of Kinoshita (20) and Greenstein (16). The glutathione concentration in the hepatomas in the present experiments is, however, lower than that found by these investigators. This point, therefore, requires a word of explanation. The experimental details differed in several important respects in the three investigations: (*a*) strain of rat; (*b*) use of primary hepatoma nodules in the present experiments as against transplanted hepatomas in Greenstein's experiments; (*c*) use of the more specific glyoxalase method for determining glutathione in the present experiments; and (*d*) possibility of a greater amount of necrosis in the hepatomas used in the

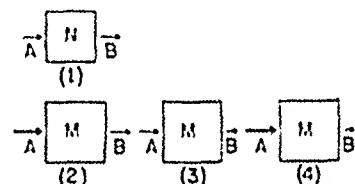


Fig. 4.—Protein metabolism in normal and malignant cells.

present experiment. In the necrotic portions of tumors the sulphydryl concentration is reported (16) to drop nearly to zero. We have been careful, therefore, to choose only the peripheral, grossly non-necrotic portions of the hepatoma nodules for study. Pieces from each hepatoma were saved for histological study. While there was microscopic evidence of some degree of necrosis present in nearly every hepatoma, necrosis was inconspicuous in all but a few sections studied. This latter seems, therefore, to be an unlikely explanation of the glutathione differences.

The primary hepatoma was used, since it affords the advantage of providing both malignant and control tissue of the same genetic origin, growing under similar environmental circumstances.

In a previous paper (31) the activity of catheptic enzymes in 30 per cent glycerol extracts of *p*-dimethylaminobenzene-induced primary hepatomas was studied. In such experiments cysteine was invariably added to the test solutions in order to provide maximal activation of the enzymes. Thus the experiments were designed to uncover possible differences in the concentration of the protein part of the enzymes in the hepatomas and control livers. Under these conditions the activity of catheptic trypsinase (benzoylcar-

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The glutathione concentration of normal and malignant tissues has been the subject of consider-

able study, as pointed out in a recent review (28). Fewer comparisons, however, have been made between glutathione concentrations of malignant tissues and their homologous normal counterparts (14, 16, 20). The present findings are in qualitative agreement with those of Kinoshita (20) and Greenstein (16). The glutathione concentration in the hepatomas in the present experiments is, however, lower than that found by these investigators. This point, therefore, requires a word of explanation. The experimental details differed in several important respects in the three investigations: (*a*) strain of rat; (*b*) use of primary hepatoma nodules in the present experiments as against transplanted hepatomas in Greenstein's experiments; (*c*) use of the more specific glyoxalase method for determining glutathione in the present experiments; and (*d*) possibility of a greater amount of necrosis in the hepatomas used in the

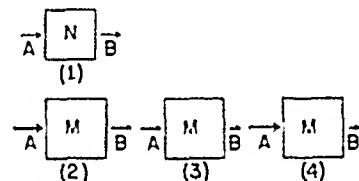


FIG. 4.—Protein metabolism in normal and malignant cells

present experiment. In the necrotic portions of tumors the sulphydryl concentration is reported (16) to drop nearly to zero. We have been careful, therefore, to choose only the peripheral, grossly non-necrotic portions of the hepatoma nodules for study. Pieces from each hepatoma were saved for histological study. While there was microscopic evidence of some degree of necrosis present in nearly every hepatoma, necrosis was inconspicuous in all but a few sections studied. This latter seems, therefore, to be an unlikely explanation of the glutathione differences.

The primary hepatoma was used, since it affords the advantage of providing both malignant and control tissue of the same genetic origin, growing under similar environmental circumstances.

In a previous paper (31) the activity of catheptic enzymes in 30 per cent glycerol extracts of *p*-dimethylaminobenzene-induced primary hepatomas was studied. In such experiments cysteine was invariably added to the test solutions in order to provide maximal activation of the enzymes. Thus the experiments were designed to uncover possible differences in the concentration of the protein part of the enzymes in the hepatomas and control livers. Under these conditions the activity of catheptic trypsinase (benzoylcar-

gininamidase) was found to be increased in the hepatoma. In the present experiments the focus has been directed toward the other side of this problem, namely, the concentration of cathepsin activators in hepatomas as compared with control livers. Thus in the hepatoma there is an increased concentration of the protein part of a catheptic enzyme but a decreased concentration of the activator of that same enzyme.

SUMMARY

The cathepsin-activating ability of ultrafiltrates prepared from hepatomas has been found to be considerably less than that of various types of control livers. Since sulphydryl groups are the most common cathepsin activators found in tissues, we have looked for a possible correlation of the above finding with the glutathione concentrations of the hepatomas. The hepatoma ultrafiltrates had both the lowest glutathione concentrations and the least cathepsin-activating power. The fetal livers, however, had a low glutathione concentration but the greatest cathepsin-activating ability. It is evident, therefore, that the cathepsin may be activated by more than one tissue component.

Glutathione appears (in the normal liver, in the regenerating liver, and in the hepatoma) to be a major cathepsin-activating agent and to be particularly low in the hepatoma. It is thus possible that the increased growth rate of this type of hepatoma may be related to a decrease in the concentration of the glutathione component of one of its protein-degrading mechanisms.

ACKNOWLEDGMENT

The authors wish to express their indebtedness to Drs. Joseph C. Aub and Ivan D. Frantz, Jr., for valuable suggestions and criticisms, and to Mrs. Carolyn Stewart for technical assistance.

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A Chemical Investigation of Keratin and Carcinomas Deriving from Rabbit Papillomas (Shope)*

HENRY W. SCHERP, PH.D., AND JEROME T. SYVERTON, M.D.†

(From the Department of Bacteriology, University of Rochester School of Medicine and Dentistry, Rochester 7, New York)

In 1933, Shope (14) described a virus-induced papilloma occurring naturally in the cottontail rabbit (*genus Sylvilagus*) and transmissible to the domestic rabbit (*genus Oryctolagus*). Later, Rous and Beard (13) found that the experimentally induced papillomas in domestic rabbits often progressed to carcinomas; and Syverton and Berry (16, 17) observed the same phenomenon in cottontail rabbits. Because of its possible significance for the pathogenesis of mammalian cancers in general, this virus-induced papilloma-to-carcinoma sequence aroused wide interest and has been the subject of numerous investigations.

An outstanding feature of the Shope papilloma is the production of excessive amounts of keratinized epithelium, which may develop into enormous epithelial horns, sharply demarcated from the underlying tissue.¹ The present communication concerns some of the chemical properties of this "keratin" and also of carcinomas deriving from the papillomas in cottontail rabbits.

Keratinization, or cornification, which reflects the ultimate natural alterative change in superficially situated structures of ectodermal origin, yields a wide variety of hard anuclear entities, such as horns, hoofs, nails, hair, the outer layers of scales, feathers, and horses' burrs. The same process occurs excessively when structures of ectodermal origin are subjected to unusual stress. Thus callosities result from excessive physiological stress and hyperplastic keratoses from physical or chemical agents, such as prolonged exposure to sunlight (8), and from carcinogenic agents (9). More striking, perhaps because of the abnormal and irregular features, are the bizarre manifestations of the process of keratinization which occur during the patho-

genesis of epitheliomas. For example, keratinized "pearls" are regularly present in cystic papillomas and acanthomas.

It has long been customary for histologists to employ "keratinization" as a descriptive term in the process of development of all corneous excrescences and to designate the resulting substance as "keratin," irrespective of whether the product was a horn, hoof, callosity, or nail. Modern chemical investigations, however, have made possible a separation into two main categories: eukeratin and pseudokeratin. Block and Vickery (2) revised the chemical criteria for the characterization of keratins when they stated: "A keratin is a protein which is resistant to digestion by pepsin and trypsin, which is insoluble in dilute acids and alkalies, in water and in organic solvents, and which, on acid hydrolysis, yields such quantities of histidine, lysine and arginine that the molecular ratios of these amino acids are respectively approximately as 1:4:12." Subsequently, Block (4) was responsible for their separation when he presented evidence that "the proteins of ectodermal origin fall into two main groups, eukeratins and pseudokeratins. The eukeratins are insoluble and resistant to digestion by pepsin and trypsin and yield histidine, lysine and arginine in molecular ratios of approximately 1:4:12. The pseudokeratins appear to be somewhat more soluble and less resistant to enzymatic digestion and yield relatively more histidine and less arginine than the eukeratins."

The pseudokeratins analyzed at that time contained histidine, lysine, and arginine in average molecular ratios of 1:3:3. In more recent studies, ratios of 1:4:6 were found (6), and it was also stated that "from 25 to 60 per cent of the pseudokeratins is often dissolved by treatment with pepsin and trypsin." According to the foregoing criteria, representative eukeratins are various animal hairs, fingernails, and cattle horn, and representative pseudokeratins are human skin, neurokeratin, and horses' burrs.

The present experiments indicate that the horny excrescence formed in rabbit papillomatosis

* This investigation was aided by a grant from the Jane Coffin Childs Memorial Fund for Medical Research.

† Now located at the School of Medicine, University of Minnesota, Minneapolis, Minnesota.

¹ As examples of the size attained by virus-induced papillomas on the ears of rabbits, we have observed two specimens that measured 13 cm. in length and more than 3 cm. in diameter and two others that measured more than 10 cm. in diameter and 6 cm. in length.

(Shope) must be classified with the pseudokeratins; its water-insoluble protein was from 32 to 51 per cent soluble in sodium hydroxide at pH 12; was insoluble in hydrochloric acid at pH 1; was from 57 to 87 per cent digested by pepsin at pH 1.8 or trypsin at pH 7.9; and contained histidine, lysine, and arginine in average molecular ratios of 1:2.3:2.5.

For comparison, specimens of the carcinomas that arose secondary to the initial virus papillomas in 6 cottontail rabbits were analyzed for the basic amino acids, to determine whether these occurred in ratios that would characterize the tissue. On the average, these materials contained relatively less histidine and a slightly higher proportion of lysine to arginine, the average molecular ratios of histidine:lysine:arginine being 1:3.3:3.1. These values were not deemed, however, to differ significantly from those found for the papilloma pseudo-keratin.

EXPERIMENTAL

Papilloma.—The keratinized portions of virus papillomas (Shope) experimentally induced on domestic or cottontail rabbits were dissected as completely as possible from the mesodermal tissues. Each preparation comprised specimens from 6 to 9 rabbits. Preparation 2, from cottontail rabbits, was dried *in vacuo* over phosphorus pentoxide immediately after collection; the moisture content was 27 per cent. The other preparations were stored in acetone until used, when the acetone was drained off and the tissue dried *in vacuo*, ultimately over phosphorus pentoxide. The dried samples were comminuted in a meat-grinder and were separated by a 40-mesh screen into fine and coarse fractions.

Ether-extracted samples were prepared in a Soxhlet apparatus. For water extraction, samples were stirred for 15 minutes in 25 volumes of water, while heating in a boiling water bath, and were then collected by centrifugation. This treatment was repeated three times, after which the material was washed with acetone and dried *in vacuo*.

Carcinoma.—Each preparation comprised material from a single cottontail rabbit. Numbers 1 through 4 were primary carcinomas that developed at the site of experimentally induced virus papillomas. Number 5 consisted of metastases in the iliac lymph nodes; No. 6, of metastases in the lungs. The specimens had been stored for various periods of time in a refrigerator in 50 per cent glycerol and buffered at pH 7.0, under vaseline seal. Each was extracted three times with approximately fifty times its volume of ethyl alcohol, then

treated similarly with ether, and finally dried *in vacuo*.

Solubility of crude papilloma keratin.—The "fine" fraction of preparation No. 2 was used without further treatment. It contained 1.4 per cent of ash, calculated as sodium, and 14.7 per cent of nitrogen (all determinations of nitrogen were made by a micro-Kjeldahl procedure), calculated on the anhydrous, ash-free basis. Then 250-mg. samples of the air-dry keratin (=33.9 mg. of nitrogen) were extracted for 24 hours at 23°–26° C. with 25.0-ml. portions of distilled water, 0.10 N sodium hydroxide, and 0.10 N hydrochloric acid, respectively. The extracts were clarified in an angle centrifuge and analyzed for total and nonprotein nitrogen, i.e., nitrogen soluble in 5 per cent trichloroacetic acid. Protein nitrogen was calculated as the difference between total nitrogen and nonprotein nitrogen. The results, summarized in Table 1, showed

TABLE I
SOLUBILITY OF CRUDE PAPILLOMA KERATIN

SOLVENT	NONPROTEIN NITROGEN DISSOLVED		PROTEIN NITROGEN DISSOLVED	
	Mg.	Per cent*	Mg.	Per cent†
Water	4.85	14	2.83	11
0.1 N NaOH	7.52	22	12.7	47
0.1 N HCl	6.75	20	6.28	23

* Percentage of the total nitrogen of the keratin.

† Percentage of the total protein nitrogen of the keratin. Total nitrogen of sample = 33.9 mg. Total nonprotein nitrogen of sample = 7.1 mg. Total protein nitrogen of sample (by difference) = 26.8 mg.

that 11 per cent of the protein of the crude tissue was soluble in distilled water, 47 per cent in the sodium hydroxide, and 23 per cent in the hydrochloric acid. Twenty-one per cent of the nitrogen of the tissue was nonprotein, a quite high value, which suggests tissue breakdown (14).

Digestibility of crude papilloma keratin.—A portion of 0.500 gm. of the fine fraction of preparation No. 2 was treated with trypsin (4 [TU]^{**} [12] of Fairechild trypsin purified according to the method of Anson and Mirsky [1]) in 30.0 ml. of 0.1 M phosphate buffer at pH 7.8. Another portion of 0.500 gm. was treated with 0.050 gm. of pepsin (Merck, U.S.P.) in 30.0 ml. of 0.050 N hydrochloric acid. As controls, samples of casein (Kuhlbaum, prepared according to Hammarskjöld) were treated in the identical manner. Digestion was carried out at 37.5° C., sterility being maintained by thymol. Samples were withdrawn immediately and after 1, 3, and 22 hours, and were analyzed for nonprotein nitrogen. The results, summarized in Table 2, showed that the keratin was extensively digested by the enzymes and, in the case of pepsin, at the same rate as casein. The more rapid digestion of casein by trypsin was probably attributable to the fact that the casein was in solution, whereas with

pepsin the undigested casein, like the keratin, remained insoluble.

Solubility and digestibility of ether- and water-extracted papilloma keratin.—Since preliminary experiments had shown that the crude papilloma keratin contained considerable lipid and water-soluble material, additional tests of solubility and digestibility were made on preparations of the tissue that had been subjected to extraction with ether and hot water (4). One hundred-milligram samples of the keratin, dried to constant weight at 100° C. *in vacuo* over phosphorus pentoxide, were extracted for 48 hours at room temperature, respectively, with 10.0 ml. of 0.1 N NaOH (final pH = 12.0) and 10.0 ml. of 0.15 N HCl (final pH = 1.0); and at 37.5° C. with 10.0 ml. of 0.5 per cent aqueous pepsin solution (Merck, U.S.P., initial pH = 1.9) and 10.0 ml. of 0.5 per cent aqueous

were carried out by the microbiological method of Stokes, Guinness, Dwyer, and Caswell (15). Each value recorded (Table 4) represents the mean of values determined in duplicate or triplicate at each of two or more levels of the sample and, in three-fourths of the cases, the results of replicate runs. The results showed a surprising uniformity in the keratins tested, which comprised one batch from domestic rabbits (6d) and the "coarse" and "fine" fractions of a batch from cottontail rabbits (7d and 7e). The average molecular ratios—histidine: lysine: arginine = 1:2.3:2.5—again approximated those reported for pseudokeratins (4). Evidently the content of the basic amino acids reflected no generic difference between the keratins, nor did screening result in any fractionation. Furthermore, extraction with hot water did not change the molecular ratios (preparations 6d_w, 7d_w, 7e_w), although the

TABLE 2
TRYPTIC AND PEPTIC DIGESTION OF CRUDE PAPILLOMA KERATIN AND OF CASEIN

TIME (hours)	TRYPSIN			PEPSIN			CASEIN		
	Total (mg.)	Keratin NPN*	Casein NPN formed by digestion	Total (mg.)	Keratin NPN by digestion	Casein NPN formed by digestion	Total (mg.)	Keratin NPN formed by digestion	Casein NPN formed by digestion
0	26.6	22.5	21.1	...	10.4
1	51.0	24.4	36	83.4	60.9	86	51.8	30.7	44.0
3	60.8	34.2	51	84.6	62.1	87	59.8	38.7	40.2
22	70.4	43.8	65	84.3	61.8	87	68.9	47.8	51.6
									73

*NPN = nitrogen soluble in 5 per cent trichloroacetic acid.

† Percentage of the total nitrogen of the keratin or casein, respectively.

trypsin solution (Fairchild, initial pH = 7.9). The enzymatic digestions were protected by thymol against contamination. The residues were collected in an angle centrifuge, washed three times in from 10 to 15 ml. of water, and dried to constant weight at 100° C. *in vacuo* over phosphorus pentoxide. (The weight of the sample minus the weight of the residue equals the weight of material dissolved or digested, respectively.)

The results, which are summarized in Table 3, confirmed the findings with the crude keratin. Although the water-insoluble material was not soluble in the hydrochloric acid, it was from 32 to 51 per cent dissolved by the sodium hydroxide and was from 57 to 87 per cent digested by the enzymes.

Determination of basic amino acids.—In an early experiment, papilloma preparation No. 2 was analyzed by Block's procedure (5) for histidine, lysine, and arginine, which were found in respective molecular ratios of 1:4.2:2.9 (Table 4), values approximating those that had been reported for pseudokeratins (6).

With one exception, to be discussed presently, all other determinations of the basic amino acids

residual proteins did contain slightly increased absolute amounts of the basic amino acids.

Comparative analyses of six papilloma-induced carcinomas revealed a fair degree of uniformity, histidine varying from 1.88 to 2.67 per cent, lysine from 5.75 to 7.26, and arginine from 6.17 to 8.10. Except for No. 1, the carcinomas contained relatively less histidine and a slightly higher proportion of lysine to arginine. The mean molecular ratios correspond relatively closely to those noted above for the keratinized portion of papillomas, a result that may reflect the common epidermal derivation of the two types of tissue.

Comparison of results with previously reported analyses of keratins.—In the first of the present series of determinations, a sample of ether-extracted hair from an albino rabbit was included as a control representing presumably a typical eukeratin. The amounts of arginine (8.80 per cent) and lysine (2.83 per cent) found were in good agreement with those reported in the most recent compilation of revised data on eukeratins (7), but the value for histidine (1.80 per cent) was nearly three times the mean value for six types of animal hair

(0.65 per cent). The basic amino acid content of rabbit hair has not been reported previously.

Because of the discrepancy in the histidine value, in later experiments measurement was made of the basic amino acid content of the same rabbit hair, of rabbit toenails (not previously reported), and of human fingernails (3), all prepared by repeated extraction with hot water and digestion by pepsin. This treatment effected a slight reduction in the histidine and increases in the lysine and arginine contents of the rabbit hair. Indeed, the arginine and lysine contents of all three specimens were somewhat higher than those previously reported for eukeratins, although the molecular ratio of arginine to histidine, 2.6, was in good agreement with the published figures, which ranged from 2.1 to 3.4, with a mean value of 2.8 for fourteen eukeratins (7). The histidine contents, however, were of the order of twice as great as expected.

Since the previously reported analyses of kera-

tins for basic amino acids had nearly all been made by some variant of the classical Kossel-Kutscher procedure (11), it seemed important to analyze one of the present specimens by one of these methods. Accordingly, a pool of preparations 6d_w, 7d_w, 7e_w was analyzed by Block's procedure (5). The arginine value found was 90 per cent of that found by the microbiological assay; the lysine value, 96 per cent; the histidine value, only 75 per cent. Furthermore, the histidine fraction was not subjected to purification by mercury and copper treatments, which of themselves may result in an additional loss of 25 per cent of the histidine present (5).

Taken together, the foregoing results support the conclusion of Vickery and Winternitz (19) that the various modifications of the Kossel-Kutscher procedure give low values for histidine. That the results may be excessively low in proteins of low histidine content is indicated by published analyses of silk fibroin, whose histidine content was re-

TABLE 3
SOLUBILITY OF PAPILLOMA KERATIN

MATERIAL*	PRELIMINARY TREATMENT†	SOLUBILITY IN		DISSOLUBILITY BY	
		0.1 N NaOH Per cent	0.15 N HCl Per cent	Pepsin Per cent	Trypsin Per cent
DR-papilloma 6d _w	Ac, E, W	51	2	82	81
CR-papilloma 7d _w	Ac, E, W	39	2	57	86
CR-papilloma 7e _w	Ac, E, W	32	0	86	87

* DR, domestic rabbit; CR, cottontail rabbit.

† Ac, extracted with cold acetone; E, extracted with ether (Soxhlet); W, extracted with hot water.

TABLE 4
BASIC AMINO ACID CONTENT OF KERATINIZED PAPILLOMAS (SHOPE), OF CARCINOMAS
AND OF SOME EUKERATINS

MATERIAL*	PRELIMINARY TREATMENT†	HISTIDINE (%)	LYSINE (%)	ARGININE (%)	Hist.	MOLECULAR RATIOS
		(%)	(%)	(%)		Lys. Arg. Arg./Lys.
CR-papilloma 2‡	None	1.48	5.93	4.77	I	4.2 2.0 0.7
DR-papilloma 6d	Ac, E	2.23	4.91	5.95	I	2.9 2.4 1.0
DR-papilloma 6d _w	Ac, E, W	2.47	5.48	6.97	I	2.4 2.5 1.1
CR-papilloma 7d§	Ac, E	2.27	4.81	6.36	I	2.3 2.5 1.1
CR-papilloma 7d _w	Ac, E, W	2.45	5.40	7.02	I	2.3 2.5 1.1
CR-papilloma 7e	Ac, E	2.34	4.64	6.16	I	2.1 2.3 1.1
CR-papilloma 7e _w	Ac, E, W	2.49	5.42	6.90	I	2.3 2.5 1.1
CR-papilloma 6d _w , 7d _w , 7e _w pooled¶	Ac, E, W	1.85	5.20	6.28	I	3.0 3.0 1.0
CR-carcinoma 1	Al, E	2.67	5.75	8.00	I	2.3 2.7 1.2
CR-carcinoma 2	Al, E	2.10	7.26	8.10	I	3.7 3.4 0.9
CR-carcinoma 3	Al, E	1.88	6.94	7.61	I	3.9 3.6 0.9
CR-carcinoma 4	Al, E	1.90	5.96	7.16	I	3.3 3.4 1.0
CR-carcinoma 5	Al, E	1.93	6.81	6.17	I	3.7 2.8 0.8
CR-carcinoma 6	Al, E	2.27	6.55	6.01	I	3.1 2.7 0.9
DR-hair	E	1.80	2.83	8.80	I	1.7 4.4 2.6
DR-hair	E, W, P, W	1.67	3.15	9.60	I	2.0 5.1 2.6
DR-toenails	W, P, W	1.26	3.47	10.40	I	2.9 7.4 2.5
Human fingernails	W, P, W	1.06	3.33	10.40	I	3.3 8.7 2.6

* CR, cottontail rabbit; DR, domestic rabbit.

† Ac, Al, E, W, extracted with acetone, alcohol, ether, and hot water, respectively; P, digested with U.S.P. pepsin at pH 1.8 and 37° C. for 24–48 hours.

‡ Analyzed by Block's procedure (5). Histidine fraction purified by mercury and copper treatments. Histidine isolated as the flavimine. Duplicate determinations.

§ "Coarse" fraction.

|| "Fine" fraction.

¶ 0.801 gm. of 6d_w; 0.901 gm. of 7d_w; 0.861 gm. of 7e_w. Analysis by Block's method. Histidine fraction not purified by mercury and copper treatments. Histidine isolated as the nitrate. Single determinations.

ported by Vickery and Block (18) to be 0.07 per cent, whereas Stokes *et al.* (15) and Guirard *et al.* (10), using different microbiological procedures, found 0.41 per cent.

SUMMARY

The horny excrescence formed in virus papillomatosis of the rabbit (Shope) appears to be a "pseudokeratin": its water-insoluble protein was from 32 to 51 per cent soluble in sodium hydroxide at pH 12; was insoluble in hydrochloric acid at pH 1; was from 57 to 87 per cent digested by pepsin at pH 1.8 or trypsin at pH 7.9; and contained histidine, lysine, and arginine in average molecular ratios of 1:2.3:2.5.

In comparison, the carcinomas that arose secondary to the initial virus papillomas contained relatively less histidine and a slightly higher proportion of lysine to arginine, the average molecular ratios of histidine:lysine:arginine being 1:3.3:3.1. The differences, however, are not deemed to be sufficient to characterize the carcinomatous tissue.

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Effects of an Antireticular Cytotoxic Serum on the Brown-Pearce Carcinoma of the Rabbit*

DAVID MOVITZ, M.D., † OTTO SAPHIR, M.D., AND ALFRED A. STRAUSS, M.D.

(From the Department of Pathology, ‡ Michael Reese Hospital, Chicago 16, Illinois)

The spleen, representing a dense portion of the reticulo-endothelial system, is apparently capable of exerting a defensive reaction to malignancy. The comparative rarity of primary splenic tumors and of metastases to the spleen (1, 2, 3, 4) directed attention to this. Metastases to the spleen occur only in association with diffusely distributed metastases involving several organs (5), although a recent interpretation of similar data emphasized no significance to this phenomenon (6). Emulsified splenic tissue has exhibited an inhibitory effect when mixed with tumor upon transplantation, particularly with splenic tissue obtained from animals either bearing or having recovered from a malignancy (7, 8, 9).

Of various homologous adult tissues grafted onto chick embryos simultaneously with heteroplastic tumor, only spleen or marrow prevented the usual growth of the tumor (10). The successful defense of the chick embryos to heteroplastic tumor implants appeared to be associated with the stromal reaction which consisted essentially of an infiltration of lymphocytes (10). Varying morphologic patterns of neoplasms, particularly their stromal relationships, reflect to some extent their biologic degree of malignancy (8, 11, 12). The importance of the stroma is further emphasized by some studies on the mechanisms of x-ray therapy of tumors (13, 14). Reticulo-endothelial reaction to malignancy is frequently observed in regional lymph nodes as follicle and/or reticulum-cell hyperplasia. With more extensive carcinomatous involvement, a more general reaction is often manifested by blood cellular changes, such as leukocytosis, with an increase in both the neutrophiles and monoeytes and a decrease in lymphocytes, eosinophiles, and basophiles (15, 16).

Additional evidence of defensive reactions of the spleen and other parts of the reticulo-endothelial system to neoplastic growth is obtained from experiments with animals either splenectomized

(17, 18, 19) or having their reticulo-endothelial system blocked by trypan blue or emetine (20, 21, 22, 23), as these procedures, properly done, were associated with either enhancement of malignancy or loss of tumor immunity.

Functionally, the reticulo-endothelial system is depressed in human beings and animals with malignancy, as is indicated by the Congo red index (24) or indirectly by the effect of administering certain extracts of their blood upon the Congo red index of normal test animals (25, 26, 27). This depression also occurs following successful tumor transplantation (26), carcinogenic x-irradiation (28), or application of carcinogenic hydrocarbons (29). Noncarcinogenic hydrocarbons similarly applied do not depress the reticulo-endothelial dye-absorbing function (30).

To augment or stimulate the defensive reactions of the reticulo-endothelial system against malignancy, several general methods have thus far been used, including splenic tissue extracts (17, 31, 32, 33, 34, 35). Concentrated homologous normal spleen extracts (33), and particularly an extract of spleen from tumor-regressed animals (34), appear to be unusually effective. Heterologous splenic extract has been administered with success to human beings with basal cell carcinoma (35). Extracts of liver (35), fetal skin (36), or human connective tissue (37) have also been used.

A second general method augmenting the defensive reactions of the reticulo-endothelial system to malignancy has been the application of tumor antibodies. Homologous Brown-Pearce tumor antibodies, developed by intraperitoneal injections of a saline extract of the tumor, suppressed the growth of intramuscularly implanted tumor fragments incubated in the specific antibody-containing immune serum prior to their transplantation (38). Administration of a specific tumor anti-serum to rats with Murphy rat lymphosarcoma resulted in a greater incidence of regressions (39).

Also, reticulo-endothelial mechanisms occurring in certain inflammations appear to be effective against a variety of lesions, including carcinoma. Various neoplasms in man have been reported to

* Aided by a grant from the Simon Sehoff Fund.

† Ira Frank Research Fellow in pathology.

‡ This department is in part supported by the Michael Reese Research Foundation.

have regressed during or subsequent to acute bacterial infections, particularly erysipelas, as first observed by Fchleisen (40). Bacterial toxin treatment, consisting of various prepared mixtures of toxins of the *Streptococcus cysipclatus* and of the *Bacillus prodigiosus* (41, 42), has given way to the purified tumor-toxic fractions of the *B. prodigiosus* (43, 44). Experiments with the anti-tumor toxin of *Schizotrypanum cruzi* by Roskin have indicated that, by itself, it is not effective upon the tumor because trypan blue blockade of the reticulo-endothelial system rendered the toxin ineffective (45).

Attempts to stimulate the reticulo-endothelial system directly by a reticulo-endothelial cytotoxic serum followed certain fundamental developments. First was the elicitation and demonstration of tissue-specific serum antibody cytotoxins against tracheal epithelium of cattle by Von Dungen; these cytotoxins were found also to be species specific (46). Next Metchnikoff demonstrated that, with minute doses of a cytotoxin, stimulatory instead of cytotoxic effects may be obtained (47). More recently Chew, Lawrence, and Stephens observed marked depression of peripheral blood and tissue leukocytes following administration of a certain dosage of antileukocytic serum to guinea pigs (48). Finally, the application of established principles of serologic titration by complement fixation to a tissue antiserum was made by Marchuk (49), who did this with a combined spleen and marrow-tissue antiserum, designating it as "anti-reticular cytotoxic serum." Using this antiserum, Bogomolets reported that either an enhancement or an inhibition of carcinoma resulted, depending on whether relatively large or small doses were administered (50).

Pomerat and Anigstein, having produced a similar antiserum, confirmed by *in vitro* study both its tissue and its species specificity. Its specific inhibitory action on spleen tissue in culture occurred with dilutions of homologous antiserum up to 1:20 (51, 52). The Walker rat sarcoma 319 in conjoint tissue culture with splenic tissue grew unimpeded. This sarcoma in culture with the homologous antiserum grew unimpeded in serum dilutions above 1:4 but was impeded in serum dilutions up to and including 1:4. The addition of the antiserum to a conjoint tissue culture of spleen and the sarcoma, however, resulted in distinct tumor inhibition in as high serum dilution as 1:256 (53). Pomerat and Anigstein further observed that relatively large doses, 0.5 cc., of the reticulo-endothelial immune serum administered to *Bartonella* carrier rats resulted in a recurrence of the *Bartonella* infection, as also occurred with

trypan blue blockade of the reticulo-endothelial system or by splenectomy (54).

Miale, studying dog spleen antiserum, observed upon its administration to dogs both a relative and an absolute increase of the mononuclear cells in the peripheral blood, which began 24 to 48 hours following the first injection and gradually returned to the base level within 20 days (55). Using the capacity for absorption of stain by histiocytes as an index of reticulo-endothelial activity, Provenzale observed that the greatest number of histiocytes per microscopic field were filled with stain in sections from spleen, marrow, liver, and lymph node from rabbits given antireticular cytotoxic serum, as compared with rabbits given an antimuscular cytotoxic serum or normal serum (56).

EXPERIMENTAL EFFECTS OF ARC¹ SERUM ON THE BROWN-PEARCE CARCINOMA

The antineoplastic properties of antireticular cytotoxic serum were investigated *in vivo* by us with rabbits implanted with Brown-Pearce carcinoma. This carcinoma has already been influenced by various procedures: immunity to the tumor was attained following intracutaneous tumor implantation (57); successful transplantation was prevented by first incubating the tumor fragments in immune serum (38); and its malignancy was lowered by exposing the tumor-bearing rabbits either to continuous illumination or to continuous darkness (58). Moreover, enhancement of its malignancy has been observed following administration of trypan blue (22), total ablation of the thyroid gland (59), or interruption of the cervical sympathetic chain (60). Abrogation of immunity to this tumor has been accomplished by trypan blue administration (23).

The Brown-Pearce carcinoma of the rabbit ordinarily exhibits a distinct natural variation in its degree of malignancy when transplanted into similar rabbits simultaneously, malignancy varying from rapidly fatal to slowly growing or even completely regressive (61). Thus this neoplasm lends itself to experimental changes. Furthermore, a well-mixed tumor suspension, with obviously identical growth potentialities throughout the suspension, when simultaneously transplanted into similar rabbits in equal quantity and into the same type of tissue (testis) will reflect the various degrees of resistance to the tumor by the resulting degree of malignancy in these rabbits. Though we used hybrid rabbits, the controls as well as the serum-treated were apparently similar hybrids.

¹ ARC=antireticular cytotoxic.

The validity of using hybrids is elucidated in the discussion.

The serum was made by the intravenous administration of increasing doses of normal spleen and marrow suspension-extract in saline to a dog at 5-day intervals until the complement fixation titer was at least 1:80 (62).

Nine consecutive experimental series were conducted, each consisting of ten or twelve hybrid male rabbits with positive intratesticular transplants of the Brown-Pearce carcinoma. The rabbits in each series were about equally divided into a serum-treated and a control group.

PROCEDURE OF TRANSPLANTATION

Tumor tissue was obtained under aseptic precautions through an abdominal incision from a previously transplanted rabbit. This was well minced and then emulsified through a tissue press.

tripled in quantity. In the last three series each dose of serum was the same and was administered at 7- to 9-day intervals. Administration of the serum was begun as soon as the tumor appeared established, usually on the fifth to tenth day following the transimplantation, except for Series II and III. In Series II the serum was begun simultaneously with the transplantation, and in Series III the serum treatment was completed 1 week prior to the transplantation. All serum was administered subcutaneously in the back and, for accuracy of dosage, in dilutions of saline ranging from 1:10 to 1:1000, depending on the undiluted dosage which ranged from 0.0001 to 0.27 cc. The serum dilution was always made immediately prior to administration. The controls received no injections whatever.

The essential data consist of (1) the duration of survival following the tumor transimplantation

TABLE 1
MALIGNANCY GRADING AS DETERMINED BY TUMOR DISTRIBUTION

Tissue involved	Level	Tissue involved	Level
Testes and peritoneum along vas	1	Plus lung (macroscopic)	6
Plus great omentum	2	Plus mediastinum	7
Plus parietal peritoneum (wall and dia-phragm) and serosa	3	Plus myoendrium and pericardium	8
Plus liver	4	Plus spleen	9
Plus kidney	5	Plus adrenal	10

Sufficient sterile normal saline was added to render the emulsified tumor tissue adequately fluid for delivery through a syringe and an 18-gauge needle. The quantity of saline necessary varied somewhat. The ratio of tumor to saline in one series, for example, was 2.5 gm. of tumor to 1 cc. of saline.

The tumor suspension was injected into both testes of all the rabbits of a series, 2 cc. of the tumor suspension being injected into each testis. During the injection, a small quantity of the suspension was felt by palpation to be escaping into the peritoneal cavity. As a result, the transplantation was principally intratesticular and to a minor extent also intraperitoneal.

Following transplantation the rabbits were placed in their cages in the animal room, which had windows on two sides and was constantly ventilated. The diet consisted of liberal portions of hay, grains, and cabbage. The testes were examined daily until the tumor appeared grossly to be growing within at least one testis. This occurred in from 5 to 10 days. The rabbits with tumors were then separated more or less equally into a serum-treated and a control group.

The serum in the first six series was administered in three doses, at about 48-hour intervals, with each successive dose being approximately

(2) the level of malignancy as based on tumor distribution found at necropsy, and (3) percentage of regressions. Degrees or levels of malignancy of this tumor were originally noted by Brown and Pearce (61) and by Pearce and Van Allen (58, 60). They defined three general levels of malignancy, the more diffuse distribution of the tumor as the more highly malignant. In the course of our Series I, it became apparent that the distribution of the tumor among the controls differed markedly from that among the serum-treated. These serum-treated animals, which also had died earlier, presented a much more diffuse distribution of the tumor. Thereupon, need for detailed biologic grading of the malignancy became apparent and was set up accordingly. High degree of malignancy appears to consist of an orderly successive involvement of organs by the tumor, as indicated in Table 1, correlating fairly well with duration of survival.

All the rabbits dying with tumor presented involvement of at least the testes and the peritoneum. The peritoneal regions presenting tumor were, first, along one or both vasa, then upward upon the great omentum and, third, on the abdominal side of the diaphragm, the mesentery, the parietal peritoneum, and frequently the serosa.

These various involvements are graded 1, 2, and 3 (Table 1).

Microscopic involvement of the lungs was noted in practically all the rabbits, even with the relatively low malignancy levels of 3. Macroscopic lung nodules, however, were found only in those tumor rabbits with a much higher level of malignancy—in those with at least liver and kidney involvement also.

Involvement of the liver in addition to the peritoneal surface was graded 4. With the kidney involved additionally, a level of 5 was designated. If in addition to involvement of the preceding regions the lung presented gross tumor nodules, a level of 6 was designated. Higher levels are further indicated in Table 1. All organs were examined histologically.

Occasionally an organ in the successive order of malignancy levels was found not to be involved. The total level of malignancy was then considered to be one degree less. Very occasionally, two or three organs were "missed," and thereupon the grading was reduced by the number of levels omitted.

This grading is apparently consistent also with the observations of Foulds, who found that trypan blue administration to rabbits with Brown-Pearce carcinoma was followed by a much higher incidence of metastases to the lungs, liver, and spleen, particularly the latter (22). Also in man, when the spleen is involved with metastatic carcinoma, it is only in association with diffusely distributed metastases to other organs, such as lung and liver (5), indicative of a highly malignant state.

Differences between serum-treated and control animals are valid only within each series, inasmuch as the degree of malignancy of this tumor appears to vary with the season and the weather (61). Also, since the quantity of exposure to light appears to influence the degree of malignancy of the Brown-Pearce carcinoma (58), the rabbits in the two groups of each series (serum-treated and controls) were so arranged in their cages in the animal room that approximately equal intensity and duration of natural and artificial light fell upon all animals of each series. In each series one group of hybrid rabbits was set up against a similar control group. The narrow range of malignancy levels occurring in each series, particularly of the control groups, attests to the adequate control of the various experimental factors—even to the extent that often a distinct variation in the levels of malignancy did not occur.

Though the levels of malignancy, for convenience, are indicated in numerical terms (Table

1) and averages of groups are calculated with these numbers, differences in malignancy between rabbits or groups of rabbits are not at all quantitative but, rather, qualitative. Thus a level of 7 is not twice as malignant as 3.5 but indicates involvement of certain organs in addition to the peritoneal surfaces. Statistical analysis does not lend itself to qualitative differences, and thus small numerical differences are more significant than would be indicated on a quantitative basis. From another point of view the concept which is suggested is that of relative organ resistance or susceptibility to metastatic neoplasia.

From theoretical considerations gained from each experimental series and applied to succeeding series for their testing, it appears that the dosage of the serum plays an essential role in the result, thus apparently confirming the observations of Bogomolets (50), of Strauss, Horwitz, Levinthal, Cohen, and Runjavac (64), and of Miale (55), and lending further support to the original attempt by Metchnikoff to stimulate cellular proliferation with minute doses of a cytotoxin (47). The interval of administration of any particular dosage also appears to be important.

SERIES I, II, AND III

In Series I, following establishment of the "testicular" tumors 9 days following transimplantation, four rabbits were given the antireticular cytotoxic serum and five served as controls. Three doses—0.03, 0.09, and 0.27 cc.—freshly diluted in 1:10 saline, were administered subcutaneously at 48-hour intervals. An apparent enhancement of the malignancy occurred following administration of the serum (Table 2). The administered dosages were thus considered cytotoxic.

In Series II the serum administration was begun 1 day after transplantation of the tumor. Half the twelve animals received three doses—0.01, 0.03, and 0.09 cc.—in 1:10 saline, at 48-hour intervals. This is one-third the dosage given in Series I, reduced because the previous dosage appeared cytotoxic, but still in the range of hundredths of a cubic centimeter. An apparent enhancement of the malignancy again occurred among the serum-treated (Table 3). The earlier administration of the serum, almost simultaneous with the transplantation, did not appear to alter its effects.

In Series III the three doses of serum were completed 1 week prior to the tumor transplantation. The dosages and intervals were the same as in Series II, and again a cytotoxic effect was apparently obtained, though less striking (Table 4).

SERIES IV, V, AND VI

Because the doses of serum in the first three series—0.01 to 0.27 cc.—were apparently cytotoxic and resulted in enhanced malignancy, the dosage of the serum in the next three series—Series IV, V, and VI—was reduced in general to one one-hundredth (1/100) of the previous quantities used.

In Series IV the transplanted tumors appeared established 10 days after the transplantation, and thereupon three doses of serum were given to half the animals at 48-hour intervals. The three doses were 0.00015, 0.0003, and 0.0009 cc., respectively, given in 1:1000 saline, freshly diluted. Again the serum-treated exhibited a higher malignancy.

Serum administration to half the rabbits of Series V was begun on the tenth day following the transplantation, at which time the tumors ap-

peared established. Three doses—0.0003, 0.0006, and 0.0009 cc., respectively—were given subcutaneously at 3- to 4-day intervals in 1:1000 saline. No significant difference was noted between the serum-treated and the controls. The malignancy levels averaged 3.75 and 3.5 for the two groups. The average survival times in the two groups were 34.5 and 30.5 days (Table 5).

Similar management of ten tumor rabbits in Series VI again resulted in no significant difference between the serum-treated and the controls (Table 9).

In view of the experience with Series V and VI, the doses of the serum ranging from 0.0001 to 0.0009 cc. were considered too minute to have an effect and thus were considered to be noneffective doses. In contrast, the doses given in the first three series, ranging from 0.01 to 0.27 cc., consistently enhanced the tumor malignancy and thus were considered to be cytotoxic doses.

TABLE 2
SERIES I

Rabbit no.	SERUM-TREATED Malignancy level	Days of survival	Rabbit no.	CONTROLS Malignancy level	Days of survival
104	7	20	112	3	26
107	7	22	102	3	27
111	7	24	108	3	43
101	7	27	106	3	44
Average	7.0	23.2	109	5	44
			Average	3.4	36.8

TABLE 3
SERIES II

Rabbit no.	SERUM-TREATED Malignancy level	Days of survival	Rabbit no.	CONTROLS Malignancy level	Days of survival
121	8	16	118	3	17
117	6	21	126	3	17
122	6	21	120*	3	38
124	5	22	125	5	62
116	5	49	119	4	64
Average	6.0	25.8	Average	3.6	39.6

* Only rabbit not showing microscopic tumor foci in lung.

TABLE 4
SERIES III

Rabbit no.	SERUM-TREATED Malignancy level	Days of survival	Rabbit no.	CONTROLS Malignancy level	Days of survival
129	6	23	130	5	68
132	5	38	137	Regressed
130	8	44	138	Regressed
131	Immune (natural)	136	Immune (natural)
Average	6.3	35	Average	5	68

SERIES VII, VIII, AND IX

A short time prior to the beginning of our Series VII, Strauss, Horwitz, Levinthal, Cohen, and Runjavac reported that a reticulo-endothelial cytostimulatory effect was obtained in the treatment of experimental fractures in rabbits with one administration of 0.00125 cc. of the antireticular cytotoxic serum (64). This dosage, 0.00125 cc., is approximately one-tenth more than each of the non-effective doses given in our Series IV, V, and VI and is approximately one-tenth of each of the cytotoxic doses given in our Series I, II, and III. Because our problem apparently required counteraction to a more or less sustained process-carcinoma, more than a single administration of serum seemed necessary.

In Series VII two doses of the serum, 0.00125 cc. each, were given, the second 9 days following the first dose, both in 1:100 saline dilution. The first dose was administered on the seventh post-

transplantation day, at which time the tumors appeared grossly established. Table 6, Series VII and Table 9 indicate the results, which show for the first time in this study a suggestion of a favorable effect by the difference in malignancy levels obtained with serum treatment.

In Series VIII three doses of serum were administered, each 0.00125 cc., at 7- and 9-day intervals, respectively. The first dose was given on the eighth day following the transplantation, at which time the "testicular" tumors became grossly established. The results again suggest a favorable effect of the serum. The malignancy level of 5.5 (serum-treated) as compared to 6.8 (controls) was associated with a favorable survival time of 40.2 days for the serum-treated as compared to 29.8 among the controls. Two regressions occurred in the serum-treated group and one among the controls (see Table 7, Series VIII, and Table 9).

In Series IX two doses of the serum, the first

TABLE 5
SERIES V

Rabbit no.	SERUM-TREATED Malignancy level	Days of survival	Rabbit no.	CONTROLS Malignancy level	Days of survival
155	3	18	157	3	18
154	3	34	161	3	18
156	5	39	160	3	24
162	4	47	159	5	62
158	Regressed	163	Regressed
Average	3.75	34.5	Average	3.5	30.5

TABLE 6
SERIES VII

Rabbit no.	SERUM-TREATED Malignancy level	Days of survival	Rabbit no.	CONTROLS Malignancy level	Days of survival
321	5	17	328	5	15
326	3	19	324	7	19
323	4	20	320	7	20
327	5	21	329	7	21
319	3	24	322	8	22
Average	4.0	20.0	318	5-	26
			Average	6.5	20.5

TABLE 7
SERIES VIII

Rabbit no.	SERUM-TREATED Malignancy level	Days of survival	Rabbit no.	CONTROLS Malignancy level	Days of survival
341	6	32	336	6	17
342	6	32	331	7	20
339	9	41	343	6	22
340	1	56	333	7	38
337	Regressed	334	8	52
338	Regressed	335	Regressed
Average	5.5	40.2	Average	6.8	29.8

at 7 days post-transplantation, and the second dose 9 days later, were given to the serum-treated animals. Each dose was 0.001 cc., given in 1:100 saline dilution. A slightly less malignant course occurred among the serum-treated, as indicated by an average 5.2 malignancy level for the serum-treated as compared to 6.1 for the controls, by an average survival time of 27.7 days among the serum-treated and 21.5 days for the controls, and by two regressions occurring among the serum-treated, whereas no regressions occurred among the controls of this series (Table 8, Series IX; Table 9).

In summary, in the first three series, the dosages of serum administered ranged from 0.01 to 0.27 cc.

and controls were 3.5 to 4.0, and the survival times of each group within each series were similar (Table 5 and Table 9). This dosage was considered to be ineffective.

Continuing by trial and error with respect to dosages and intervals in treatment, the dosage of the serum in Series VII, VIII, and IX was increased up to one-tenth of the cytotoxic doses, 0.001 cc., and in addition was administered only every 7 to 10 days instead of at 48-hour intervals. An apparent tumor-inhibitory effect was observed in the serum-treated groups in these last three series (Table 9). In Series VII the serum-treated malignancy level was 4.0 as compared to 6.5 for

TABLE 8
SERIES IX

Rabbit no.	SERUM-TREATED		Rabbit no.	CONTROLS	
	Malignancy level	Days of survival		Malignancy level	Days of survival
352	4-	19	350	6-	16
353	5-	19	358	5	16
354	9	24	349	6	21
356	3	49	361	7	21
351	Regressed	.	363	6	21
355	Regressed	.	362	7	34
Average	5.2	27.7	Average	6.1	21.5

TABLE 9
SUMMARY

SERUM DOSAGE	SERIES	MALIGNANCY LEVEL Serum-treated (average)	CONTROLS Serum-treated (average)	DAYS OF SURVIVAL Serum-treated (average)	CONTROLS (average)	REGRESSIONS (PER CENT) Serum-treated (average)	CONTROLS (average)
0.01 to 0.27 cc.	I	7.0	3.4	23.2	36.8	0	0
	II	6.0	3.6	25.8	39.6	0	0
	III	6.3	5.0	35	68	0	66
0.00015 to 0.0009 cc.	IV	5.0	3.3	24	40.3	40	40
	V	3.75	3.5	34.5	30.5	20	20
	VI	3.5	4.0	26	24.6	60	40
0.001 cc.	VII	4.0	6.5	20	20.5	0	0
	VIII	5.5	6.8	40.2	29.8	33	16
	IX	5.2	6.1	27.7	21.5	33	0

Each serum-treated rabbit was given three increasing doses, at 48-hour intervals. An enhancement of the malignancy occurred with these dosages, as is summarized in Table 9. In each of these first three series a higher level of malignancy and a shorter survival time were noted in the serum-treated groups. Hence these dosages were considered cytotoxic, or too large.

Therefore, in the next three series—Series IV, V, and VI—approximately one one-hundredth of the previous quantities of serum was administered, the doses ranging from 0.0001 to 0.0009 cc. Except for Series IV, in which the serum-treated again exhibited an enhanced malignancy, no significant difference between the serum-treated and the controls was noted (Table 9). In Series V and VI the levels of malignancy for both serum-treated

the controls. In Series VIII the serum-treated level was 5.5 as compared to 6.8, and the survival time of the serum-treated also was comparatively favorable, 40.2 days as contrasted to 29.8 days for the controls. In Series IX the malignancy level of the serum-treated was 5.2 as compared to 6.1 for the controls. The difference in survival time, though small, is also in favor of the serum-treated, 27.7 to 21.5 days. Furthermore, the regression rates in Series VII and VIII also tend to suggest a less malignant course following administration of the serum.

DISCUSSION

Our results would seem further to substantiate two concepts. First, neoplasia may be affected by reticulo-endothelial tissue, inasmuch as adminis-

tration of a reticulo-endothelial tissue antiserum, previously shown to affect reticulo-endothelial tissue (51, 52, 55, 56) affected the malignancy of the Brown-Pearce carcinoma. Second, a cytotoxic serum may be given in doses that apparently produce cytostimulation instead of cytotoxic effects, although, essentially, a tissue antiserum is cytotoxic and its biologic identification in the past has depended on its cytotoxic exhibition. It is clearly realized, however, that direct observation of any reactions of the reticulo-endothelial system to the antiserum were not made. The reactions are presumed because cited evidence indicates an inverse relationship between stimulative reaction of the reticulo-endothelial cells and tumor growth, i.e., enhanced malignancy being associated with depression of this tissue system and, conversely, inhibition of the tumor being associated with a stimulated reaction of the reticulo-endothelial system. In corroboration, the larger doses would therefore be depressing or cytotoxic (associated with enhanced malignancy), and the smaller doses would be related to the stimulation of this system and associated tumor inhibition. This appears to have occurred.

The cytotoxic effect (enhancement of malignancy) was more easily and clearly obtainable in our various series than was the cytostimulatory effect (tumor inhibition, as suggested by the results of our last three series). The difficulty in obtaining the presumed cytostimulatory effect of the reticulo-endothelial system on this carcinoma may be surmised from the fact that many and widely distributed nodules were present rather than a single lesion or one with just a few metastases. The relatively short duration of the animal's life with this tumor also indicates its high malignancy. Thus it would seem that a cytotoxic effect of this serum, with further depression of the reticulo-endothelial function upon its administration, would be markedly accentuated because of the extensive carcinomatous involvement and, conversely, that an attempt to stimulate the functions of this tissue system would be hampered by the multiplicity of the tumor lesions.

The levels of malignancy among the controls varied somewhat from time to time. In the course of the experiments during 1946 the levels among the controls were, in general, rather low, varying from 3.3 to 4.0, but mostly between 3.3 and 3.6. Beginning with the fourth series, higher levels of malignancy occurred among the controls and continued so for the remainder of the several series, varying from 6.1 to 6.8.

The use of a pure breed of rabbits would seemingly reduce the average range in the natural

variations of malignancy of the Brown-Pearce tumor. A pure strain of rabbits, however, was not available. Casey observed variations in the degree of malignancy of the Brown-Pearce tumor, by statistical averages, between a number of different pure strains of rabbits (63). However, a considerable range of variation in the malignancy within each pure strain occurred, as indicated in Fig. 1 of Casey's report (63). Gorer observed that a mouse sarcoma which had arisen in a pure-breed line required very specific genes for its progressive growth, which, consequently, limited its successful transplantation to this particular pure breed or to certain of its hybrids (66). In contrast, Casey's experience indicates that the Brown-Pearce tumor, arising presumably in a hybrid ("albino male of stocky build"), was readily transplantable into, and lethal for, almost all varieties of pure strains (63) and hybrid rabbits. This would indicate a widespread occurrence of the particular genes necessary for the progressive growth of the Brown-Pearce tumor, although these have not as yet been determined. These widely occurring genes would also tend to obviate the necessity for using pure strains. The hybrids used in our various series appeared to serve as excellent controls, exhibiting insignificant variations, particularly as regards the narrow range of the degrees of malignancy as noted in the controls of the several series. Since Gorer has shown, by transplantation with the sarcoma arising in a pure-breed mouse, that some of the genes necessary for the progressive growth of a transplanted tumor may be identical with those determining blood groupings (66), it is possible that determinations of blood groups would be helpful in explaining some variations in the degree of malignancy among the different animals of a species.

Another *in vivo* demonstration of obtaining opposite effects with identical reticulo-endothelial tissue antiserum simply by varying the dosage was made in a study of this serum on experimental bone fractures in rabbits by Strauss, Horwitz, Levinthal, Cohen, and Runjavac (64). The quantity of serum which stimulated healing of fractures was 0.00125 cc., given once. This identical dosage, though repeated once or twice at 7- to 10-day intervals, was the same which we found apparently to stimulate reticulo-endothelial processes against malignancy.

Rogoff, Freyburg, Powell, and Rice reported that no effect of antireticular cytotoxic serum was obtained when it was administered to rats with induced pleuropneumonia arthritis (67). Inasmuch as the size of the dosage appears to be a critical factor, it is interesting to note that the dosage of

the serum that they employed, 0.00015 cc., was the same dosage with which we also could not, in general, detect a difference between the serum-treated and the controls (Ser. V and VI).

The variations in levels of malignancy within each group (serum-treated and controls) was small, fluctuating only slightly. However, marked variations in the levels of malignancy in Series VIII and IX occurred among the serum-treated. This might be interpreted as the result of the dosage of serum being in a critical zone between cytostimulatory and cytotoxic, in accordance with each individual animal's reticulo-endothelial reactivity to the amount of serum given. This would indicate that a reticulo-endothelial functional guide, similar in principle to the Congo red index test, might be helpful in determining the individual dosage necessary to obtain a reticulo-endothelial cytostimulatory effect.

SUMMARY

Administration of a reticulo-endothelial tissue antiserum, previously known to affect reticulo-endothelial tissue directly by *in vitro* tissue culture, was followed by definite effects on the degree of malignancy of the Brown-Pearce carcinoma *in vivo*. Reticulo-endothelial cytotoxic serum may be given in doses that apparently produce stimulation instead of a cytotoxic effect. The cytotoxic effect of the serum on the Brown-Pearce carcinoma was indirectly and presumably well demonstrated in the first three series by producing definitely enhanced malignancy. A dose too minute to have an effect was observed in Series V and VI. Finally, a mild cytostimulatory effect was obtained with one-tenth the cytotoxic dose, given at 7- to 10-day intervals, as indirectly indicated by the resulting tumor inhibition.

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In vitro Studies on the Effect of Spleen, Striated Muscle, and Kidney upon the Growth of Sarcoma 180 and Mammary Carcinoma of Mice*

LOGAN O. JONES, M.D.

(From the Department of Pharmacology, College of Physicians and Surgeons, Columbia University, New York)

The rarity of metastatic cancer to spleen, striated muscle, and kidney has been repeatedly noted in the past (1, 7, 29, 40, 63, 76), a fact which suggests that the malignant cell meets unknown influences antagonistic to its survival and proliferation in such sites. Cowdry has recently commented once again on this enigma, adding: ". . . it would be logical to try the effects of extracts of these tissues [spleen, striated muscle, and kidney] on experimental cancers . . ." (23).

Approaches to this problem employing *in vitro* technics have not demonstrated any tumor inhibition due to splenic influences. Stevenson (67), Danchakoff (24), Lumsden (50), and Wylegschanin (75) have reported observations on growth of neoplastic and splenic tissues in close proximity, either in tissue culture or following implantation into developing chick embryo membranes. In the several rodent carcinomas and sarcomas studied, tumor growth and cytology were not influenced by splenic explants from several mammalian sources, including the tumor donor strain of animals. The fact that Rous chicken sarcoma grows well in tissue culture with spleen (28) may not be germane to this problem, since a virus etiology has now been established for this tumor.

The several *in vitro* observations on concomitant growth of neoplastic and striated muscle tissue are not in agreement. Centanni (20) noted growth inhibition of a mouse carcinoma when skeletal muscle taken from tumor-resistant mice was added to cultures of this tumor but could detect no growth suppression upon addition of skeletal muscle explants from susceptible mice. In contrast, Wylegschanin (75) reported no antagonistic effects due to skeletal muscle in a similar study on a sarcoma and a carcinoma from rodent hosts. *In vitro* growth suppression of a rat sarcoma by several digests of bovine heart muscle was observed by Roffo (55). Since this worker could detect no

similar effects from fresh muscle extracts from the same source, he attributed the observed inhibition of tumor growth to the presence of free amino acids in his muscle digests.

There are few recorded results on tumor growth in tissue cultures to which kidney explants have been added. Neither Lumsden (50) nor Wylegschanin (75) could detect alterations of tumor histology when a carcinoma and several sarcomas from rodent hosts were cultivated with renal tissue from tumor-resistant rodents.

These *in vitro* findings give no hint as to why cancer apparently metastasizes so infrequently to spleen, striated muscle, and kidney, and they do not lend support to the many favorable reports of animal and human malignancy treated with various organ extracts. The fact that such observations continue to appear in the literature may indicate that some workers are unaware of negative *in vitro* and *in vivo* studies in the past. It seemed of interest to reinvestigate these older tissue-culture observations and to supplement them by inoculating susceptible hosts with tumor explants after they had been cultured with these various tissues, in order to note *in vivo* tumor growth as well. To our knowledge, such latter observations have been made previously only by Stevenson (67) in the case of tumor exposed to splenic tissue *in vitro*.

MATERIALS AND METHODS

The studies were made on two commonly employed mouse tumors: sarcoma 180 from a Swiss mouse host, and a spontaneous adenocarcinoma of the breast obtained from mice of the Paris strain. Control biopsies were obtained in each case for future comparison. Spleen, skeletal muscle, and kidney from the same strain as the respective tumor donors were used for culture.

The roller-tube tissue-culture technic of Gey was selected to allow intimate contact of tissue juices from organ explants with growing tumor explants. A few hanging-drop preparations, using

* This work was supported by a grant from the Committee on Growth, acting for the American Cancer Society.

the "flying cover slip" method of Maximov, were also made; and, when growth in such preparations was found to differ in no detectable manner from that in the roller tubes, these hanging-drop cultures were used for making photographic records and permanent histologic whole mounts. In all studies, chicken plasma clots provided the matrix for growth; and chicken embryo extract, Tyrode's solution, and human placental serum mixtures supplied nutrient factors. Simms's ultrafiltrate of ox serum (60) was incorporated into the feeding solution of the hanging-drop preparations, to supply growth stimulants and to suppress fat globules.

Nonulcerated, firm tumors, approximately 2.5 cm. in diameter, from 10- to 14-week-old mouse hosts, were selected for tissue culture. For each tumor a total of 100 viable fragments, 2 mm. square, were planted, a quarter of which were

RESULTS

No observable alterations of tumor cytology or growth characteristics over the control observations were detected in the two malignant growths studied when grown in tissue culture adjacent to spleen, skeletal muscle, or kidney explants. Growth of the normal tissue explants which were adjacent to viable tumor cells resembled that of fragments of these tissues grown alone. No less than 84 per cent of tissue and tumor explants showed good growth in all series throughout the period of tissue-culture observations (see Table 1 and Figs. 1-6).

Following reinoculation of sarcoma 180 into susceptible hosts, the percentage of "takes" with tumor explants exposed to splenic and to renal tissue in culture was lower than the control figure observed when tumor fragments cultured alone were inoculated. These percentages do not differ

TABLE 1
THE EFFECTS OF SPLEEN, SKELETAL MUSCLE, AND KIDNEY ON TUMOR GROWTH

Tissue Culture	SARCOMA 180					MAMMARY ADENOCARCINOMA				
	Tumor alone	Tumor Spleen	Tumor Muscle	Tumor Kidney		Tumor alone	Tumor Spleen	Tumor Muscle	Tumor Kidney	
Total explants	25	25+25	25+25	25+25		25	25+25	25+25	25+25	
Per cent viable explants, 10-12 days	86	96+100	100+96	92+84		96	100+100	96+96	100+100	
Growth	Good	Good	Good	Good		Good	Good	Good	Good	
Cytology	Normal*	Normal*	Normal*	Normal*		Normal*	Normal*	Normal*	Normal*	
ANIMAL INOCULATIONS	Tumor alone	"Splenic" tumor†	"Muscle" tumor†	"Renal" tumor†						
Number of animals	20	20	10	11						
Per cent "takes"	65	30	90	46						

* "Normal" connotes no observed difference from control organ or tumor in culture.

† Prefix "splenic," etc., refers to tumor explants grown with spleen, etc., in tissue culture.

grown in roller tubes in close proximity to spleen explants, another quarter with kidney explants, and a third quarter with skeletal muscle explants. Twenty-five explants of tumor and an equal number of each organ were grown separately to serve as controls. Because of the longer "lag-phase" of kidney and skeletal muscle, these explants were grown in culture for 4 to 8 days to insure good growth before starting the tumor cultures. Thus, for the neoplasms studied, the behavior of 200 explants of tumor was noted. Observations were made daily and photographic records of living and stained whole mount preparations were made at the end of 9 to 12 days. At the end of 10 to 12 days of tissue culture, sarcoma 180 tumor explants were injected into the dorsal subcutaneous tissues of the same strain as the tumor donor, and subsequent *in vivo* growth followed. Biopsy and histologic study were carried out after these tumors had reached approximately 2.5 cm. in diameter. These latter studies could not be carried out with explants of the spontaneous breast adenocarcinoma because susceptible hosts could not be obtained.

significantly, however, by statistical analysis. It is possible that the figure of 30 per cent "takes" with the "splenic" tumor explants and the 46 per cent "takes" following animal inoculation with "renal" tumor would attain significance in a larger group of animals (see Table 1). Since the time of appearance, the rapidity of growth, and the histology of the tumors did not differ in the experimental and the control groups of animals, repetition of the studies, using larger numbers of mice, was not deemed worth while.

DISCUSSION

Our inability to detect suppression of tumor growth *in vitro* attributable to the influence of spleen, skeletal muscle, or kidney is in essential agreement with the findings of previous workers. The influence of tissues of other mouse strains and of heterologous tissues was not observed, since the presumed antagonism exerted by spleen, skeletal muscle, and kidney exists in tumor hosts themselves, or metastatic cancer of these organs would

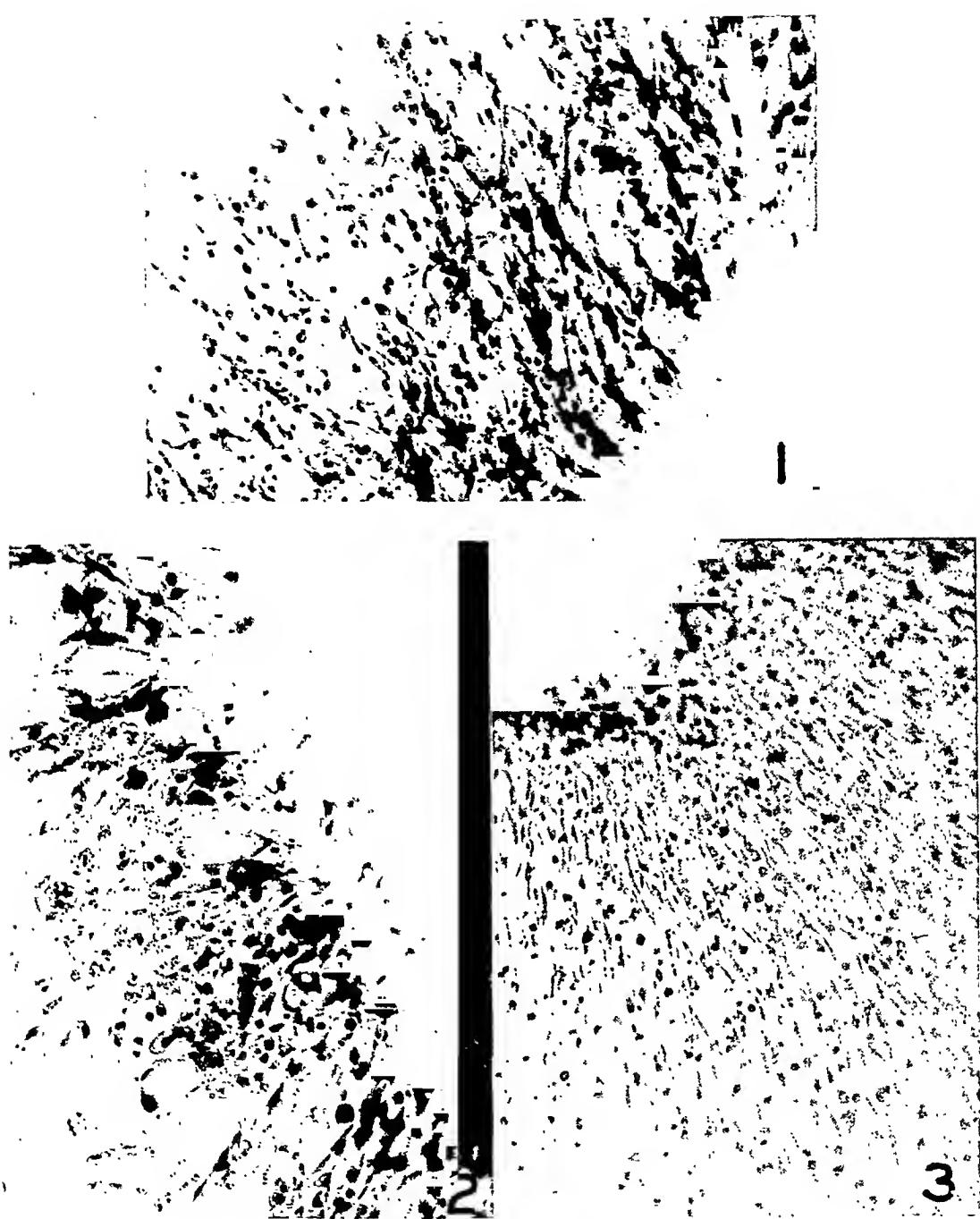


FIG. 1.—Sarcoma 180 grown alone for 10 days in tissue culture, during which time the explant was transplanted twice. Fixation in Bouin's and staining with Harris' hematoxylin. $\times 103$.

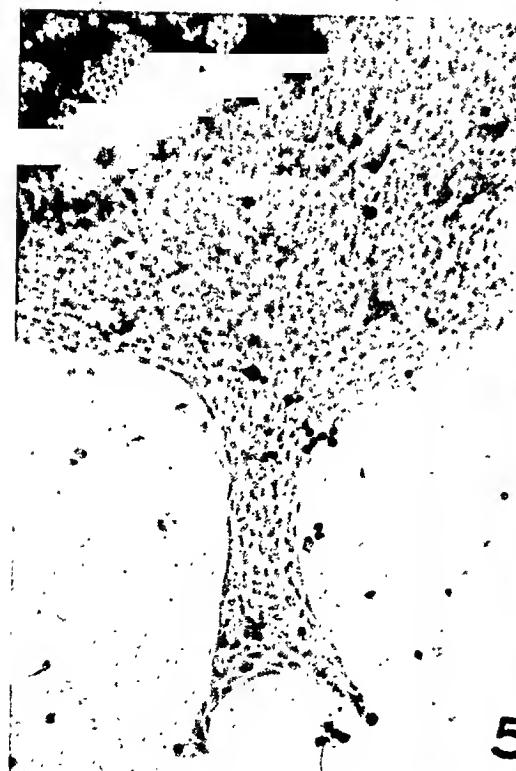
FIG. 2.—Sarcoma 180 grown with spleen for 10 days in tissue culture, during which time it was transplanted

twice. Fixation in Bouin's and staining with Harris' hematoxylin. $\times 103$.

FIG. 3.—Mouse spleen grown with tumor depicted in Fig. 2. Fixation in Bouin's and staining with Harris' hematoxylin. $\times 103$.



4



5



6

FIG. 4.—Spontaneous mammary adenocarcinoma of the mouse grown in tissue culture alone for 9 days. Fixation in Bouin's and staining with Harris' hematoxylin. $\times 103$.

FIG. 5.—Spontaneous mammary adenocarcinoma of the mouse grown for 9 days in tissue culture with kidney.

Fixation in Bouin's and staining with Harris' hematoxylin. $\times 103$.

FIG. 6.—Mouse kidney grown with tumor depicted in Fig. 5. In this case the kidney was grown alone for 4 days to insure good growth, then transplanted at the time the tumor was planted. Fixation in Bouin's and staining with Harris' hematoxylin. $\times 103$.

be more common. The enthusiastic reports of *in vitro* studies in which tissue extracts have been used as therapeutic agents may bear closer scrutiny in the light of *in vitro* studies, since we feel such enthusiasm has often been unwarranted. The use of tissue extracts in the chemotherapy of malignant disease will be presented only in outline form, in an attempt to interpret the rationale for their use and to analyze results. More extensive reviews (66, 71, 73, 74) of this subject bring out one fact worth emphasis at the start, namely, that consistently reproducible results have not been obtained with tissue derivatives. It is almost superfluous to add that no one of these preparations enjoys widespread clinical use—which is cogent testimony that they are inactive against malignant disease in humans.

A. DERIVATIVES OF SPLEEN AND RETICULO-ENDOTHELIAL SYSTEM

The use of splenic extracts in cancer therapy is inextricably bound to the use of various preparations to stimulate the reticulo-endothelial system, and this, in turn, to attempts to immunize actively and passively against cancer. Early reports by Woglog (70), Brannstein (13), and Levin (41) that splenic tissue implants and autolysates suppressed tumor growth in animals were essentially confirmed by others (32, 33, 34, 45). Subsequent attempts to prepare more potent compounds from splenic sources have continued to the present (2, 5, 10, 25, 46, 64, 69). It is worth emphasis that during such studies (25, 65, 69) stimulation of tumor growth and carcinogenic activity have been noted when splenic extract preparations were used.

The splenic hypertrophy and lymph node enlargement in some animals with malignant disease have been interpreted as activation of host defenses. Those proponents of the theory that the reticulo-endothelial system exerts suppressive influences upon tumor cells emphasize the frequency of splenic and lymph node hypertrophy in tumor hosts and point out that blockade of the reticulo-endothelial cells by foreign substances (3, 47, 56), their suppression by x-ray (51), or splenectomy (15, 52) has been associated with decreased animal resistance to cancer. Caspari (19), Braunstein (14), Fichera (27), and Bogomolets (11) have claimed that stimulation of the reticulo-endothelial system by their several preparations was an effective form of cancer therapy. These various observations require analysis. First, splenomegaly and lymphadenopathy are only inconsistently seen in malignant disease. When present, plausible coexistent infectious causes for reticulo-endothelial hypertrophy in tumor-bearing hosts can frequently be

demonstrated by bacteriologic methods (72). Second, we are unaware of any reports indicating that humans splenectomized for blood dyscrasias, infections of the spleen, or traumatic rupture of that organ have a higher incidence of cancer than does the general population. Third, several workers (31, 42, 53, 54) have carried out successful inoculation of the intact animal spleen with a variety of animal tumors. All the *in vitro* evidence on adjacent growth of splenic and tumor tissue previously outlined is contrary to the hypothesis that extracts of the spleen may suppress the growth of existent cancer in animals. Finally, the favorable clinical and laboratory reports of several workers using reticulo-endothelial stimulant preparations in malignancy have not been confirmed by others (6, 18, 61). From the data presented in a very recent report (62) the favorable conclusions seem unwarranted.

Because the reticulo-endothelial system has been considered to have a significant function in immune responses in infectious diseases, the phenomenon of acquired or natural resistance to cancer was studied in relation to this system. The inhibition of tumor growth by sera of immune animals reported by some (21, 35, 44, 48) could not be confirmed *in vivo* (57) or *in vitro* (39) by other workers. The enthusiastic reports (4, 9, 38, 43, 49, 64) of immunizing animals against malignancy by the use of various products of actual tumor origin find no confirmation by other workers (22, 30, 36). In the case of certain animal tumors for which a virus etiology seems assured, humoral antibodies have been detected capable of conferring cancer resistance (17, 26, 37, 58). That viral agents play a role in the genesis of human cancer or in the majority of animal tumors has not yet been demonstrated, however. Immunity or resistance to neoplastic disease cannot be attributed to the presence of circulating antibodies on the basis of the evidence now available. That antagonistic influences differing from true antibodies and originating in the reticulo-endothelial system are responsible for natural or acquired resistance to cancer has not been shown. No morphologic evidence of splenic tissue stimulation by proliferating tumors has been detected *in vitro*. These objections can be justly raised, we feel, to the use of various "tumor vaccines" and to antireticular cytotoxic serum (ACS).

B. PRODUCTS OF STRIATED MUSCLE

The evidence that striated muscle extracts act as cancerolytic agents is rather limited. Early work by Itami (34) suggested that cardiac muscle extracts but not skeletal muscle preparations were capable of immunizing animals against subsequent

tumor inoculation. Over a period of years Roffo and his associates employed various muscle derivatives in experimental cancer therapy. They observed (55) first that fresh muscle extracts were inactive but that an acid hydrolysate of bovine heart caused regression of sarcomatous and carcinosomatous lesions in rodent hosts when injected into the tumor or at remote sites. Boyland later reported (12) that an orally administered acid extract of ox heart suppressed tumor growth in rodents. Roffo attributed the activity of his preparation to the presence of free amino acids, while Boyland presented evidence that certain organic bases were the active ingredients in his muscle digests causing tumor regression. Since Itami inhibited cancer growth by using watery extracts of cardiac muscle, his conclusions are weakened by reports of these later workers. His negative results with skeletal muscle extracts are in agreement with our *in vitro* observations. No explanation based on differences in chemical composition between cardiac and skeletal muscle can be offered to account for his observation of tumor inhibition by cardiac but not by skeletal muscle extracts. Available analyses of amino acid content of these two types of striated muscle show almost identical qualitative and quantitative results (8). Studies by Shear (59) and by Turner (68) in which alleged cancerolytic effects of various chemical compounds were re-investigated do not support the hypotheses of those who had contended that free amino acids and certain organic bases in striated muscle were active cancerolytic agents. After *in vitro* studies of tumor growth in several types of media, Brues and his associates (16) attributed a minimal tumor-inhibiting effect to ethanalamine, an observation previously recorded by Boyland in his *in vivo* studies. For these reasons we submit that more work is needed before definite conclusions can be drawn as to the efficacy of striated muscle derivatives in the treatment of malignant disease.

C. PRODUCTS OF KIDNEY

When he mixed rodent carcinoma cells with renal extracts prior to animal inoculation, Frankl (32) noted poor subsequent growth of tumor. There seem to have been few similar studies until Sperti reported (64) that watery extracts of kidney tissue enhanced cancer susceptibility in mice. He further stated, however, that deproteinized, cell-free extracts from the same source were capable of immunizing animals against tumor proliferation. To our knowledge this work has not been repeated by others. The *in vitro* studies previously mentioned, including our own, have failed uniformly to detect the presence of antagonistic influences to

tumor growth inherent in kidney tissue. It would seem that *in vivo* studies bear repetition or that the negative results of those who have unsuccessfully attempted them should be recorded.

Several facts seem worth emphasis in conclusion. First, there is a dearth of reports confirming the efficacy of various reticulo-endothelial stimulants and derivatives of spleen, skeletal muscle, and kidney in the therapy of cancer. When such products have been used in human malignancy, most attempts to corroborate initial enthusiastic reports have been unsuccessful. Second, in most of the laboratory work in the field of organotherapy of malignant disease, observations have been made on transplantable animal tumors. It is a commonly accepted fact among cancer research workers that such transplantable neoplasms often undergo spontaneous involution and regression, thus making interpretation of *in vivo* results more difficult. Lastly, all *in vitro* observations of which we are aware show no depression of tumor growth by spleen or by kidney tissue. In the case of striated muscle extracts, *in vitro* observations are not in entire agreement. The allegedly low incidence of splenic, striated muscle, and renal metastases in malignancy remains unexplained.

SUMMARY

1. Mouse sarcoma 180 and spontaneous adenocarcinoma of the mouse mammary gland were grown in tissue culture with homologous spleen, skeletal muscle, and kidney from nontumor-bearing animals.
2. No tumor-growth effect was detected when such series were compared with tumor explants grown alone in tissue culture.
3. After tissue culture with spleen, skeletal muscle, and kidney, explants of sarcoma 180 produced malignant growths in mouse hosts histologically similar to control biopsies. The time of appearance and growth characteristics of these tumors did not differ from neoplasms appearing in control animals inoculated with tumor explants grown in tissue culture alone.

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One subcutaneous injection of the following hormones was made each week: 16.6 µgm. of alpha-estradiol benzoate in oil, 1.25 mg. of testosterone propionate in oil, or 1 mg. of progesterone in oil.² Daily subcutaneous injections of 25 I.U. of pregnant mare's serum (PMS) were made in other mice. The number of animals in the several groups of experiments are given in Table 1. The experiments extended for a period of 10 months.

Vaginal smears were obtained from ovariectomized mice at intervals during the experiments. At autopsy the ovarian grafts and visceral organs were examined and preserved for histological

largest tumor measured 15 × 16 × 17.5 mm. (Fig. 4). The mouse that had no tumor of the grafted ovary died when the graft was 187 days old (Table 1).

The seminal vesicles and prostates of the castrated male mice bearing intrasplenic ovarian grafts were atrophic. The adrenal glands showed degenerate x-zones. No detectable hormonal effect on the kidneys and submaxillary glands was noted (8). In some animals the liver showed degenerate changes, as indicated by the large size of hepatic cells and the number of binucleated cells.

Castrated females.—Five granulosa-cell tumors and four luteomas developed in intrasplenic ovarian grafts in 9 castrated females (Fig. 1). These tumorous grafts ranged from 212 to 296 days of age, and the largest tumor measured 8 × 11 × 14 mm.

Irregular estrous vaginal smears were obtained during the latter part of the experiment in two ovariectomized mice bearing ovarian grafts that developed into granulosa-cell tumors. The liver of one (C_3H strain) of these two mice showed nodular regeneration, and its uterus weighed 135 mg. Corni-

Gonadectomized Mice with Intrasplenic Ovarian Grafts

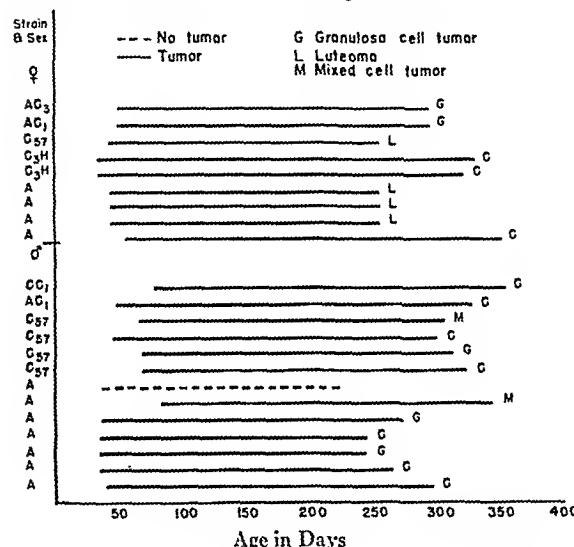


FIG. 1.—*A*, C_3H , and C_57 refer to inbred strains of mice. The other symbols designate hybrid mice derived as follows: AC_1 ($A\varphi \times C_3H\sigma$); AC_2 ($C_3H\varphi \times A\sigma$); AC_3 (C_3H low tumor $\varphi \times A\sigma$); and CC_1 ($C_57\varphi \times CBA\sigma$).

study. Some of the induced ovarian tumors were transplanted into other mice to determine their capacity for continuous growth in other hosts.

OBSERVATIONS

A. TUMORS IN INTRASPLENIC OVARIAN GRAFTS IN CASTRATED MICE

Castrated males.—Ten granulosa-cell tumors and two mixed tumors—both granulosa and luteoma cells—developed in intrasplenic ovarian grafts in 13 castrated males (Fig. 1). These tumorous grafts ranged from 207 to 261 days of age, and the

² Grateful acknowledgment is made to Dr. E. Schwenk, of the Schering Corporation, for the alpha-estradiol benzoate (Progynon-B), testosterone propionate (Oreton), progesterone (Proluton), and PMS (Anteron); and to Dr. E. Oppenheimer, of the Ciba Pharmaceutical Products, Inc., for the progesterone.

Unilaterally Gonadectomized Mice with Intrasplenic Ovarian Grafts

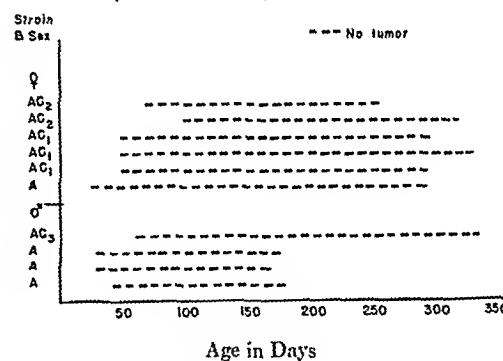


FIG. 2.—For meaning of symbols refer to Fig. 1

fied vaginal smears were not noted in the other 7 female hosts, and their uteri weighed, on the average, 45 mg. The adrenal glands showed degeneration of the x-zone.

B. OVARIAN GRAFTS IN UNILATERALLY CASTRATED MICE

Ten unilaterally gonadectomized male and female mice carried intrasplenic ovarian grafts for 138 to 282 days (Fig. 2). The ovarian grafts remained small, about 2 to 3 mm. in size. Histologically, the grafts that resided in the female hosts were composed of small and large follicles and corpora lutea (Fig. 3). No hemorrhagic follicles or tubular ingrowths of the germinal epithelium were

noted. On the other hand, the ovarian follicles and interstitial cells formed the main structures of the ovarian grafts taken from the male hosts. Little lutein tissue was encountered. Ingrowths of the germinal epithelium were observed in small areas in the oldest ovarian graft in a male mouse.

The average uterine weight of the unilaterally castrated female mice was 105 mg. The intact testes and accessory genital organs in the males were maintained in a normal condition.

C. OVARIAN GRAFTS IN ESTROGEN-TREATED CASTRATED MICE

Castrated males.—No tumors were noted in intrasplenic ovarian grafts in 8 castrated males treated with an estrogen. The treatment was started 103 to 138 days subsequent to the grafting, and most of these hosts had received more than 10 weekly injections (Fig. 5).

The ovarian grafts were 3 to 4 mm. in diameter. They contained few to many small and medium-sized follicles. The stroma was composed of interstitial cells and, frequently, luteinized interstitial cells. Proliferation and tubular ingrowths of the

germinal epithelium were observed in some areas in seven grafts, and some of the tubular structures were lined by simple cuboidal or eolumnar cells, as described previously (8). A few atretic and hemorrhagic follicles and corpora lutea were present in three grafts.

The urinary bladders of the male hosts were distended with urine. Effects of the estrogenic hormone were also indicated by the histological features of the seminal vesicles, prostates, kidneys, and submaxillary glands. The x-zones of the adrenal glands were absent, and groups of deeply stained small cells were noted in the subcapsular regions of the cortex of almost all adrenal glands examined.

Castrated females.—The intrasplenic ovarian grafts in 5 castrated females treated with an estrogen were nontumorous. The treatment was started 103 to 133 days subsequent to the grafting; 5 to 10 weekly injections were administered (Fig. 5).

Ovarian follicles and corpora lutea made up the greater part of three grafts that showed ingrowths of the germinal epithelial cells. A few hemorrhagic follicles were present in one of these grafts. Regres-



FIG. 3.—Photomicrograph of a large part of an intrasplenic ovarian graft that had persisted 246 days in a unilaterally ovariectomized mouse. Several small and medium-sized follicles are present. $\times 105$.



FIG. 4.—A small area of a large granulosa-cell tumor that had arisen in an intrasplenic ovarian graft in an ovariectomized mouse. No follicles were present in this graft. $\times 105$.

sive changes were observed in the two other grafts, although they contained ovarian stroma and few primary follicles.

The urinary bladders were also distended in the female hosts. Occasional cornified vaginal smears were obtained from one female mouse before estrogen treatment was started. The average uterine weight was 149 mg. The histological appearance of the adrenal glands resembled that of the males.

D. OVARIAN GRAFTS IN ANDROGEN-TREATED CASTRATED MICE

Castrated males.—No tumors developed in intra-splenic ovarian grafts in 8 castrated male mice that had been treated with testosterone propionate.

Gonadectomized Mice with Intrasplenic Ovarian Grafts 16 µgm. *a*-Estradiol Benzoate Injected per Week

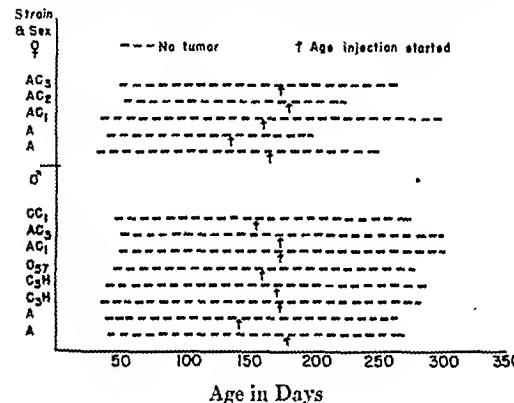


FIG. 5.—For meaning of symbols refer to Fig. 1

The treatment was started 96 to 130 days subsequent to the grafting, and 9 to 17 weekly injections were administered (Fig. 6).

The ovarian grafts ranged from 2 to 6 mm. in greatest diameter. A unique histological feature of these grafts was the increase of fibrous connective tissue in, and vascularity of, the stroma. With the exception of two degenerate grafts, they consisted largely of ovarian follicles and a few atretic and hemorrhagic follicles. No corpora lutea were noted. Tubular and cystic ingrowths of the germinal epithelium were observed in three grafts, and most of these structures contained coagulated fluid and macrophages.

The seminal vesicles and prostates of the androgen-treated male hosts were large, and their average weight was 0.35 gm. No x-zones were noted in the adrenal glands, and groups of small subcapsular cells were present in some areas. The kidneys and submaxillary glands were of the male type.

Castrated females.—Six castrated female mice, bearing intra-splenic ovarian grafts, received 13 to

17 weekly injections of testosterone propionate, starting 101 to 128 days subsequent to the grafting. The ovarian grafts were small and nontumorous (Fig. 6). Their histological structures were similar to those in the male hosts, although more luteinized interstitial cells were present in the stroma. In two grafts, germinal epithelial cells formed papillary ingrowths. Large cysts or tubular cavities were derived from ingrowths of the same type of cell. Extensive degeneration was observed in one of the six grafts.

An estrous vaginal smear was obtained in one female host before the injection of testosterone propionate was started. The average uterine weight of this group was 133 mg. Other organs exhibited evidence of androgenic effects, as in the male hosts.

E. OVARIAN TUMORS IN PROGESTERONE-TREATED CASTRATED MICE

Castrated males.—Five granulosa-cell tumors and two mixed tumors—both granulosa and luteoma cells—arose in the intra-splenic ovarian grafts in 8 castrated males treated with progesterone.

Gonadectomized Mice with Intrasplenic Ovarian Grafts 1.25 mg. Testosterone Propionate Injected per Week

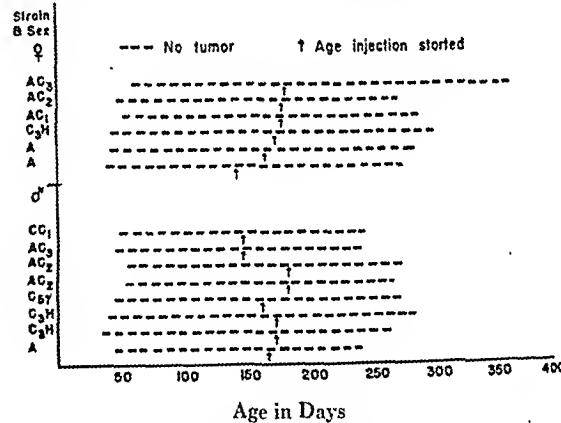


FIG. 6.—For meaning of symbols refer to Fig. 1

The treatment was started 115 to 138 days after the grafting, and 9 to 19 weekly injections were administered (Fig. 7). The largest tumor measured 14.5 × 15 × 21 mm.

In general, the granulosa-cell tumors were partially encapsulated and exhibited diffuse, folliculoid, or trabecular arrangements. Numerous mitotic figures indicated rapid growth. Some folliculoid structures were filled with blood, others with coagulated material containing a few degenerating cells. Luteinized interstitial cells were noted adjacent to small edematous or necrotic areas. Prolifer-

ation and tubular ingrowths of the germinal epithelium were present in four of these tumors. A spicule of bone developed at the periphery of the largest granulosa-cell tumor, and the tumor had metastasized to the liver. This tumor was transplanted subcutaneously into other mice of the same strain (6).

The mixed tumors were composed of masses of granulosa and luteoma cells, in addition to areas of luteinized interstitial cells and ingrowths of the germinal epithelium. The one nontumorous graft was 139 days old and consisted primarily of luteinized interstitial cell stroma and ovarian follicles. Some of the follicles were filled with blood. Four other intrasplenic ovarian grafts with vascularized adhesions were not included in this paper.

The seminal vesicles and prostates of the male hosts were atrophic. The submaxillary glands and the kidneys resembled those of castrated animals.

Castrated females.—Three granulosa-cell tumors, one luteoma, and one mixed tumor developed in intrasplenic ovarian grafts in 5 castrated females treated with progesterone. The treatment was started 127 to 134 days after the grafting; 14 to 19

Gonadectomized Mice with Intrasplenic Ovarian Grafts
1 mg. Progesterone Injected per Week

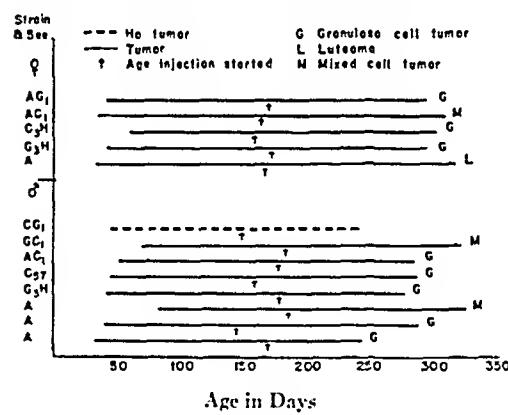


Fig. 7.—For meaning of symbols refer to Fig. 1

weekly injections were administered (Fig. 7). The largest tumor measured 12 × 15 × 17 mm.

Histologically, the granulosa-cell and mixed tumors resembled those in the male hosts, with the exception of more extensive areas of luteinized tissue and necrosis. No metastasis was noted from these tumors.

An estrous vaginal smear was observed once, before the progesterone injections were started, in a female mouse of the C₅H strain that had the largest granulosa-cell tumor, whose uterus weighed 150 mg. at autopsy. The average uterine weight of

the ether females was 41 mg. Hyperplasia of the small subcapsular cells was noted in three adrenal glands.

F. OVARIAN TUMORS IN GONADOTROPHIN-TREATED CASTRATED MICE

Castrated males.—One granulosa-cell tumor and two mixed tumors were found in intrasplenic ovarian grafts in 5 castrated males treated with daily injections of PMS, starting 100 to 119 days after the grafting (Fig. 8). The largest tumor measured 4 × 4 × 6.5 mm. (Fig. 9). One nontumorous ovarian graft was attached to the peritoneum of the body wall and the other to the small intestines.

The seminal vesicles and prostates of the male hosts were atrophic. The adrenals showed degeneration of the x-zones. The histological structure of

Gonadectomized Mice with Intrasplenic Ovarian Grafts
25 I.U. Gonadotrophin (PMS) Injected per Day

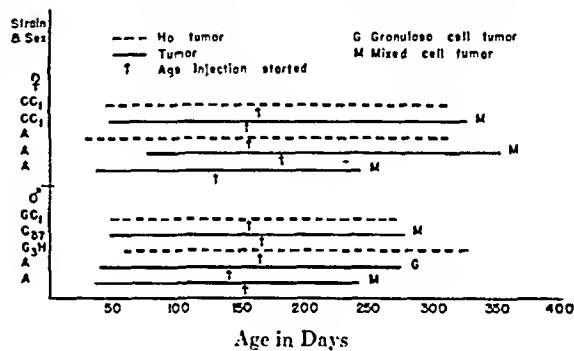


Fig. 8.—For meaning of symbols refer to Fig. 1

the kidneys and submaxillary glands was similar to that of the gonadectomized male mice.

Castrated females.—Three mixed tumors developed in intrasplenic ovarian grafts in 5 castrated females treated with daily injections of PMS, starting 92 to 127 days after the grafting (Fig. 8). The largest tumor measured 5 × 7 × 8 mm.

The histological appearance of the mixed tumors was similar to that of the male hosts similarly treated. In one tumor, spicules of bone were observed in a necrotic area adjacent to the granulosa-tumor cells. Numerous osteoblasts lined the surfaces of the bone matrix, and fibroblasts and macrophages were scattered around the osseous tissue. The two nontumorous grafts were small and consisted of a few ovarian follicles, corpora lutea, and degenerative connective tissue stroma.

Irregular estrous vaginal smears were obtained in one female before the daily injections of PMS were started; no cornified estrous smears were noted in the female hosts following treatment. The

average uterine weight was 42 mg. The x-zone of the adrenal glands was absent. As in the eunuch males, the parietal layer of the renal corpuscles were usually composed of low epithelial cells. The terminal tubules of the submaxillary glands in two animals bearing tumorous grafts, however, consisted of columnar cells.

DISCUSSION

The present experiments provide further evidence that pituitary gonadotrophic hormones are involved in ovarian tumorigenesis, at least in the



FIG. 9.—A small section of a tumorous graft that had been in the spleen of a castrate male mouse for 208 days. This mouse had received gonadotrophin (pregnant mare's serum). The area of the graft photographed shows epithelial-lined spaces surrounded by partially luteinized cells. The epithelial cords are continuous with the germinal epithelium that partially surrounds all well-developed grafts. Some of the tubules contain cells similar to those in the intertubular areas. $\times 105$.

experimental ovarian tumorigenesis in castrated mice bearing intrasplenic ovarian transplants. The failure of ovarian tumors to appear in the intrasplenic ovarian grafts in unilaterally castrated male and female mice shows that the intact gonads inhibit tumor formation in the transplants. These observations support those made previously that

intrasplicial grafts with extensive adhesions to the body wall or uterus—areas drained by other than the hepatic portal system—did not become tumorous (6, 7). Both the intact gonad as well as the transplanted gonads, if connected with the caval venous system, inhibit tumorigenesis.

Whether the steroid hormones usually presumed to be produced by the gonads—namely, estradiol and testosterone—or some other or unknown substances inhibited tumorigenesis of the transplants was not revealed by experiments of the type referred to above. The failure of ovarian tumors to appear in intrasplicial ovarian grafts in castrated mice that were given injections of estradiol benzoate or testosterone propionate indicates that the endocrine products presumably produced by the normal gonads inhibit the formation of granulosa-cell tumors and luteomas. Progesterone in the amounts used and with the weekly schedule of injection did not alter the incidence of tumors. Larger amounts might be effective. Burrows and Hoeh-Ligeti (3) failed to alter the incidence of mammary tumors in mice of the C₃H strain given weekly doses of 1 mg. of progesterone.

The observations on the group of mice that received the gonadotrophic preparation (PMS—Anteron) were inconclusive. Such preparations produce a marked luteinization of the ovaries (12, 14), and the ovaries so treated produce substances that have an androgenic effect (11, 12). At the present time it is assumed that the pituitary stimulation responsible for tumorigenesis in intrasplicial grafts in castrated mice is primarily the follicle-stimulating hormone. It is well known that the urine of postmenopausal women contains increased amounts of a substance that is primarily follicle-stimulating. The occurrence of mixed granulosa-cell tumors and luteomas in the castrate male mice indicates that the gonadotrophin did have some effect, as such tumors are noted rarely among untreated, graft-bearing male mice.

Whether or not the tumors arising in intrasplicial transplants are comparable to those arising in x-rayed mice has been questioned (4). Certainly, they possess a capacity to metastasize to the liver (4, 6), and several of them have been transplanted successfully into other and untreated hosts of the same strains. Intrasplenic ovarian grafts when irradiated did not become tumorous more rapidly than when they were not irradiated (10). The period subsequent to irradiation at which tumors appear is longer than that subsequent to transplantation into the spleens of castrate mice. If the theory that ovarian tumors are induced in irradiated mice by attainment of a hormonal imbalance proves to be correct, then the hormonal factors attained sub-

quent to irradiation must be less favorable. Irradiated ovaries do produce periodic vaginal cornification in mice; Brambell, Parkes, and Fielding (2) described estrous cycles in mice made anovular by roentgen irradiation. A low level of estrogen production may reduce the increased production of gonadotrophin and hence increase the length of the period of stimulation required.

Unlike tumors of some types, the ovarian tumors appear in mice of all strains that have been studied up to this time. Similar tumors were originally described in rats (1).

The tumors produce estrogen, as the uteri of the hosts bearing them are larger than those of castrated animals. The estrogen must be produced in amounts sufficiently large to permit some of it to pass through the liver or to be of a type not inactivated by the liver. Several animals showed hepatic damage, and one showing the most extensive indications of damage had a large uterus weighing 135 mg.

Adrenal tumors have been described in mice castrated at birth (15, 16) or at later ages (5). Strain differences in the tendency for such tumors to appear have also been described (13, 16). None of the mice in the present study had such tumors. The period of survival was not sufficiently long for them to have been expected. They have been noted, however, in x-irradiated mice (unpublished).

SUMMARY AND CONCLUSIONS

1. Granulosa-cell tumors, luteomas, and mixed tumors (granulosa and luteoma cells) developed in intrasplenic ovarian grafts in castrated male and female mice. There is apparently no strain limitation in the formation of these tumors in mice.

2. No ovarian tumors were observed in the intrasplenic grafts of ovaries in (a) unilaterally gonadectomized mice, (b) gonadectomized mice that received estradiol benzoate or testosterone propionate, or (c) gonadectomized mice with vascularized adhesion that permitted ovarian hormones to bypass the hepatic portal circulation. The gonadal hormones are assumed to act indirectly by inhibiting the production and secretion of the pituitary gonadotrophic hormones.

3. Weekly treatment with 1 mg. of progesterone did not prevent tumor formation in intrasplenic ovarian grafts.

4. Daily injections of a gonadotrophin from the

pregnant mare's serum (PMS) exerted luteinizing influence on the ovarian tumors.

5. The malignancy of the induced granulosa-cell tumors is shown by the ability to metastasize and the transplantability into new hosts.

6. These experimental results appear to substantiate further the assumption that prolonged stimulation by increased amounts of gonadotrophic hormones is responsible for the genesis of ovarian tumors.

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Dehydrogenase Studies of Tissue from Normal and Tumor-bearing Mice

I. Total Dehydrogenase Activity

Sr. M. AGATHA RIEHL, O.S.B., AND Sr. M. PETRA LENTA, O.S.B.

(From the College of St. Scholastica, Unit of the Institutum Diri Thomae, Duluth 2, Minnesota)

Evidence has been accumulated that several components of enzyme systems important in biological oxidations are altered or impaired in malignancy. While numerous reports have appeared on the dehydrogenases in normal and neoplastic tissues, many phases of the problem remain uninvestigated, and much of the earlier work needs confirmation and supplementation.

It would seem that the deficiencies reported in the various oxidative systems of tumor tissues—for example, cytochrome c and cytochrome oxidase (3, 10, 12)—would result in an accumulation of reducing substances in the tissue and that the altered oxidative mechanism would be associated with changes in the individual enzyme systems. These considerations led to the investigation of (a) the comparative ability of various tissues of normal and tumor-bearing animals to decolorize methylene blue anaerobically, the results of which are presented herewith, and (b) the comparative activity of the specific dehydrogenases of the various organs of normal and tumor-bearing animals, the results of which are presented in a subsequent report.

A decreased ability of certain types of tumor tissue to decolorize methylene blue has been reported (6, 7), but Barron did not observe such a difference (1). Reports of the influence of the presence of a tumor on the enzymatic activities of organs remote from the site of the tumor (4, 5) prompted the analysis of the dehydrogenase activity of a number of different organs in tumor-bearing animals. Furthermore, it seemed desirable to determine whether differences in susceptibility of various strains of mice to transplanted tumors were correlated with alterations in the dehydrogenase activity.

Schlenk and others (11, 2, 8) have reported that there is a reduction in the content of coenzyme I in tumor tissues. Also, as Von Euler (12) and others have shown, coenzyme I in malignant tissues is present mainly in the dihydro form, whereas in most normal tissues it exists chiefly in the oxidized state. Since this important biocatalyst is so defi-

nitely altered in neoplastic tissues, it was of interest to investigate the activity of dehydrogenase systems of normal and tumor tissues when coenzyme I was added in varying concentrations to dehydrogenase-containing extracts of various organs of normal and tumor-bearing animals.

EXPERIMENTAL

Mice of widely varying ages and both sexes of the homozygous dba (38 animals) and C57 (25 animals) strains and the heterozygous Rockland albino (28 animals) strain were used. All animals were maintained on a standard Rockland mouse-pellet diet. The tumors studied included the transplanted dbrB adenocarcinoma and sarcoma 180, as well as methylcholanthrene-induced tumors. At the time of experiment the tumors ranged between 8 and 25 mm. in diameter.

The animals were killed by decapitation, and the organs to be analyzed (i.e., skeletal muscle from the hind limbs and the spleen, liver, kidney, and brain) were immediately removed. These organs were weighed on an analytical balance and then ground with washed and ignited sand in a mortar with a small volume of cold distilled water to the consistency of a smooth thin paste, 2 ml. of water being used for each 0.1 gm. (wet weight) of tissue. Care was taken to rinse the mortar thoroughly with the last few milliliters of the calculated volume of cold water. These washings were then added to the extracted mixture, which was placed in a centrifuge tube, stirred well, and then centrifuged at 800 r.p.m. for 5 minutes. The centrifugate was removed and tested immediately for its ability to decolorize methylene blue anaerobically according to the Thunberg technic.

In measuring the dehydrogenase activity, 2 ml. of chilled tissue extract were placed in the stopper of the Thunberg tube. Into the tube proper the following reagents were introduced: 0.25 ml. of a 1:5000 methylene blue solution and 1.0 ml. of phosphate buffer, pH 7.2. The tubes were evacu-

ated with gentle agitation for 5 minutes at about 12 mm., after which the contents were brought to 37° C. in a constant-temperature water bath by allowing an equilibrium period of 10 minutes. The enzyme-containing tissue extract was then tilted into the methylene blue-buffer mixture and the decolorization time determined visually. Each test was made in duplicate. All extracts and organs were kept at 0° C. when not in use. Necrotic and hemorrhagic portions of the tumors were carefully excised and discarded before analysis. The enzyme activity was arbitrarily expressed as minutes⁻¹ × 10². With proper precautions the results were readily reproducible.

properly speaking, this is a measure of the total reducing substances present in the extract of the tissue. In calling such a reaction the "total dehydrogenase activity," it was presumed that the crude tissue extracts prepared in the manner described contained sufficient quantities of the substrates, since the addition of several substrates operating in systems requiring coenzyme I did not cause significant changes in the decolorization time by the crude extracts. In a number of cases, however, the addition of substrates not requiring coenzyme I speeded up the activity to some extent.

The reliability of the technic was shown by the fact that in 96 per cent of the duplicate or triplicate

TABLE I
DECOLORIZATION TIME OF METHYLENE BLUE BY AQUEOUS EXTRACTS OF SKELETAL MUSCLE FROM
NORMAL AND TUMOR-BEARING MICE IN THE PRESENCE AND ABSENCE OF COENZYME I

STRAIN OF MICE	COENZYME I (mg.)	No. DETERMINATIONS		DECOLORIZATION TIME (MIN.)		VELOCITY (min. ⁻¹) × 10 ²	PER CENT INCREASE BY COEN- ZYME I
NORMAL ANIMALS							
dba	.0 0	14	25.7	(20.0-33.0) (2.5-7.0)	± 4.46	3.3	
	.0 5	12	5.1	(2.5-7.0)	± 1.64	19.5	457
Rockland	.0 0	18	39.2	(21.0-56.0)	± 3.03	3.3	
	.0 5	14	2.7	(2.0-4.0)	± 0.73	37.0	1021
C57	.0 0	22	29.3	(20.0-34.0)	± 3.73	3.3	
	.0 5	18	3.3	(2.5-4.0)	± 0.45	90.3	765
TUMOR-BEARING ANIMALS							
dbrB in dba	.0 0	18	28.5	(21.0-32.0)	± 3.96	3.3	
	.0 5	18	4.4	(2.5-6.0)	± 1.33	22.7	543
Methylcholanthrene in dba	.0 0	4	23.0	(20.0-30.0)	± 5.00	4.0	
	.0 5	4	3.0	(3.0)	± 0.60	23.3	722
S 180 in Rockland	.0 0	16	27.6	(20.0-34.0)	± 3.83	3.6	
	.0 5	12	2.8	(2.0-3.5)	± 0.62	35.7	881

Coenzyme I was prepared according to the method of Williamson and Green (13). While the degree of purity of the product was not determined experimentally, comparable results could be obtained by standardizing one preparation against the next. A suitable concentration was selected for investigation of its comparative effects on the dehydrogenase activity of extracts of tissues from normal and tumor-bearing animals by adding varying amounts of the coenzyme I to the extracts of tissues from normal dba mice. The tissue extracts were so diluted that a definite alteration of dehydrogenase activity was apparent when the amount of coenzyme I was varied as determined in preliminary experiments. The same type of study was made with extracts of the adenocarcinoma dbrB and sarcoma 180.

The ability of a tissue extract to reduce methylene blue in an oxygen-free medium without the addition of substrates or coenzymes was designated as the "total dehydrogenase activity." More

determinations the total dehydrogenase activity varied by not more than 2 minutes, and the variations for organs from animals of the same strain did not exceed ± 3 minutes. The results presented in this paper represent 840 determinations.

RESULTS

There was a marked similarity of the total dehydrogenase activity of analogous organs of normal animals of different strains (Tables 1-5). The presence of the transplanted tumor did not significantly alter this activity in any of the organs studied. The order of increasing dehydrogenase activity in both normal and tumor-bearing mice of all strains was as follows: muscle, spleen, brain, kidney, and liver. Furthermore, a difference in susceptibility to the dbrB tumor in the C57 and dba strains was not reflected in the values for the total dehydrogenase activity.

There was apparently a distinct difference be-

tween dehydrogenase activity of the methylcholanthrene-induced tumor and sarcoma 180, on the one hand, and the dbrB adenocarcinoma, on the other (Table 6), although relatively few methylcholanthrene-induced tumors were available for study. This difference may have been due in part to the fact that the calculations were made on the basis of the wet weight; by dry-weight determinations the adenocarcinoma contained 85 per cent water, as compared to the sarcoma, which contained 72 per cent water.

The addition of coenzyme I accelerated the decolorization time of methylene blue by spleen extracts much less than it did the other normal tissues (Tables 1-5), and this was true also in additional experiments in which coenzyme I was added in amounts up to 1.5 mg. In unreported experiments both the adenocarcinoma and the sarcoma

180 responded markedly to the addition of coenzyme I in varying concentrations. Results from the addition of coenzymic I at a level of 0.5 mg. to extracts of tumor tissue are shown in Table 6. The percentage stimulation of dehydrogenase activity was higher than that in similar preparations of spleen (Table 2) but was lower than that of liver (Table 5), kidney (Table 4), brain (Table 3), and muscle (Table 1).

DISCUSSION

Perhaps the most significant observation to be made in these studies is the marked similarity and stability of the activity of the so-called "total" dehydrogenases. As shown by the experimental data, this ability to decolorize methylene blue was independent of animal age, sex, and strain. Furthermore, the presence of a transplanted tumor (sar-

TABLE 2

DECOLORIZATION TIME OF METHYLENE BLUE BY AQUEOUS EXTRACTS OF SPLEEN FROM NORMAL AND TUMOR-BEARING MICE IN THE PRESENCE AND ABSENCE OF COENZYME I

STRAIN OF MICE	COENZYME I (MG.)	NO. DE- TERMINA- TIONS	DECOLORIZATION TIME (MIN.)	S.D.	VELOCITY (MIN. ⁻¹ × 10 ²)	PER CENT STIMULATION BY COEN- ZYME
NORMAL ANIMALS—SPLEEN						
dba	{0.0 0.5	10 8	10.8 7.5	(9.0-13.0) (7.0-9.0)	1.47 ±0.86	9.2 13.3
Rockland	{0.0 0.5	18 10	12.1 10.0	(9.0-15.0) (7.0-12.0)	±1.79 ±1.69	8.2 10.0
C57	{0.0 0.5	8 8	11.8 9.0	(10.0-14.0) (8.0-11.0)	±1.48 ±1.23	8.4 11.1
TUMOR-BEARING ANIMALS—SPLEEN						
dbrB in dba	{0.0 0.5	22 16	12.0 9.3	(8.0-18.0) (6.0-14.0)	±3.17 ±3.56	8.3 10.7
Methylcholanthrene in dba	{0.0 0.5	6 6	12.6 8.0	(8.0-13.0) (6.0-11.0)	±2.69 ±2.24	7.8 12.5
S 180 in Rockland	{0.0 0.5	12 12	10.0 8.0	(8.0-11.0) (7.0-10.0)	±1.15 ±0.93	10.0 12.5

TABLE 3

DECOLORIZATION TIME OF METHYLENE BLUE BY AQUEOUS EXTRACTS OF BRAIN FROM NORMAL AND TUMOR-BEARING MICE IN THE PRESENCE AND ABSENCE OF COENZYME I

STRAIN OF MICE	COENZYME I (MG.)	NO. DE- TERMINA- TIONS	DECOLORIZATION TIME (MIN.)	S.D.	VELOCITY (MIN. ⁻¹ × 10 ²)	PER CENT STIMULATION BY COEN- ZYME
NORMAL ANIMALS—BRAIN						
dba	{0.0 0.5	8 8	8.5 2.0	(6.0-11.0) (1.5-2.5)	±1.80 ±0.35	11.7 50.0
Rockland	{0.0 0.5	16 16	9.8 2.4	(8.0-13.0) (2.0-3.0)	±1.61 ±0.48	10.2 41.6
C57	{0.0 0.5	8 8	10.5 2.3	(10.0-11.0) (2.0-3.0)	±0.50 ±0.37	9.5 43.4
TUMOR-BEARING ANIMALS—BRAIN						
dbrB in dba	{0.0 0.5	14 14	8.1 2.2	(6.0-13.0) (2.0-4.0)	±2.35 ±0.70	12.3 36.8
Methylcholanthrene in dba	{0.0 0.5	4 4	10.0 2.5	(10.0) (2.0-3.0)	0.00 ±0.50	10.0 40.0
S 180 in Rockland	{0.0 0.5	6 6	9.3 2.3	(8.0-10.0) (2.0-3.0)	±0.94 ±0.47	10.7 43.4

TABLE 4

DECOLORIZATION TIME OF METHYLENE BLUE BY AQUEOUS EXTRACTS OF KIDNEY FROM NORMAL AND TUMOR-BEARING MICE IN THE PRESENCE AND ABSENCE OF COENZYME I

STRAIN OF MICE	COENZYME I (mg.)	No. DETERMINA-TIONS	DECOLORIZATION TIME (MIN.)	S.D.	VELOCITY (MIN. ⁻¹ × 10 ²)	PER CENT STIMULATION BY COENZYME
NORMAL ANIMALS—KIDNEY						
dba	0.0	12	4.40	(4.0–5.0)	±0.45	22.7
	0.5	10	1.20	(0.5–1.5)	±0.40	83.3
Rockland	0.0	12	2.60	(2.0–3.5)	±0.68	38.4
	0.5	12	0.53	(0.4–0.7)	±0.10	188.6
C57	0.0	30	4.20	(3.0–6.0)	±0.89	23.8
	0.5	18	1.20	(0.7–2.0)	±0.37	83.3
TUMOR-BEARING ANIMALS—KIDNEY						
dbrB in dba	0.0	24	3.20	(2.0–6.0)	±1.08	31.2
	0.5	24	0.95	(0.4–2.0)	±0.48	105.2
Methyleholanthrene in dba	0.0	4	2.40	(2.25–2.5)	±0.17	41.6
	0.5	4	0.50	(0.3–0.7)	±0.63	200.0
S 180 in Rockland	0.0	10	2.50	(2.0–3.0)	±0.45	40.0
	0.5	10	0.73	(0.7–0.75)	±0.02	138.0

TABLE 5

DECOLORIZATION TIME OF METHYLENE BLUE BY AQUEOUS EXTRACTS OF LIVER FROM NORMAL AND TUMOR-BEARING MICE IN THE PRESENCE AND ABSENCE OF COENZYME I

STRAIN OF MICE	COENZYME I (mg.)	No. DETERMINA-TIONS	DECOLORIZATION TIME (MIN.)	S.D.	VELOCITY (MIN. ⁻¹ × 10 ²)	PER CENT STIMULATION BY COENZYME
NORMAL ANIMALS—LIVER						
dba	0.0	16	1.80	(1.5–2.0)	±0.24	55.5
	0.5	10	0.40	(0.3–0.5)	±0.10	250.0
Rockland	0.0	14	1.90	(1.1–2.0)	±0.30	76.1
	0.5	14	0.38	(0.3–0.5)	±0.07	263.0
C57	0.0	30	1.70	(1.1–3.0)	±0.31	58.8
	0.5	16	0.52	(0.3–1.0)	±0.20	192.1
TUMOR-BEARING ANIMALS—LIVER						
dbrB	0.0	24	1.80	(1.0–2.0)	±0.36	55.5
	0.5	10	0.40	(0.3–0.5)	±0.09	250.0
Methyleholanthrene in dba	0.0	4	1.90	(1.3–1.3)	0.00	76.1
	0.5	4	0.40	(0.3–0.5)	±0.10	250.0
S 180 in Rockland	0.0	10	1.30	(1.0–1.7)	±0.22	76.1
	0.5	10	0.38	(0.3–0.5)	±0.08	263.0

TABLE 6

DECOLORIZATION TIME OF METHYLENE BLUE BY AQUEOUS EXTRACTS OF TUMORS IN THE PRESENCE AND ABSENCE OF COENZYME I

STRAIN OF MICE	TUMOR TYPE	COENZYME I (mg.)	No. DETERMINA-TIONS	DECOLORIZATION TIME (MIN.)	S.D.	VELOCITY (MIN. ⁻¹ × 10 ²)	PER CENT STIMULATION BY COENZYME
dba	dbrB adenocarcinoma	0.0	20	16.8	(14.0–22.0)	±2.18	5.9
		0.5	14	6.0	(5.0–7.0)	±0.84	16.6
dba	Methyleholanthrene	0.0	4	4.1	(3.3–5.0)	±0.85	24.4
		0.5	4	1.6	(1.25–2.0)	±0.40	62.5
Rockland	Sarcoma 180	0.0	24	5.4	(4.0–6.5)	±0.76	18.5
		0.5	24	2.5	(2.0–3.0)	±0.32	40.0

coma or carcinoma) caused no change in the activity of the animal tissues. In contrast to the reports of deficiencies and changes in the oxidative metabolism of tumor tissue, suggesting an accumulation of reducing substances, the results of the present study indicate no significant increase in the amount of water-soluble materials capable of reducing methylene blue in the organs of tumor-bearing animals. Strictly speaking, comparison of the activity of tumor tissue with any except that of the analogous normal tissue is unwarranted; but it can be observed that this activity in tumor tissue showed no striking difference from that of certain nonneoplastic tissues. Tumor tissue appeared to have an activity rate intermediate between that of the more active normal liver and kidney tissue extracts, on the one hand, and the less active extracts of brain, spleen, and muscle tissue, on the other. Whether the tumor causes alterations in specific dehydrogenase systems of organs remote from the site of the neoplasm is to be the subject of future studies, but it is probable that such variations would not be detected by the methods used in the present study.

The results obtained when coenzyme I was added in varying concentrations to tumor extracts would not suggest that reducing substances were present in large enough quantities or in a sufficiently potent state to inhibit the coenzyme activity of systems containing concentrations of coenzyme I greater than those normally present in the tissue.

The failure of large amounts of coenzyme I to effect a significant stimulation of dehydrogenase activity of the spleen in both normal and tumor-bearing animals cannot be adequately interpreted at present. According to the report of Bernheim and Felsovanyi (2), the coenzyme content of spleen is slightly higher than that of liver, kidney, and muscle. If coenzyme I is normally present in excess in the spleen, additional amounts of coenzyme I would hardly result in an acceleration of activity. The possibility is not precluded, however, that certain coenzyme I-requiring dehydrogenases are present in diminished quantities or are entirely lacking. As indicated in this study, spleen extract is not the least active of extracts of normal tissues in decolorizing methylene blue, but subsequent investigations of individual enzyme systems (9) have revealed slight or no lactic dehydrogenase activity in the spleen. The malic dehydrogenase content of the spleen is also quite low, having about the same decolorization time as malic dehydrogenase in the several different tumors analyzed.

SUMMARY

Quantitative determinations of the total dehydrogenase activities of the muscle, liver, kidney,

brain, and spleen of normal homozygous dba and C57 mice and heterozygous Rockland albino mice revealed a remarkable similarity of activity among the various animal strains. The presence of a tumor (transplanted dblB adenocarcinoma, methylcholanthrene-induced tumors in dba mice, and transplanted sarcoma 180 in Rockland mice) did not alter the ability of any of the tissue extracts studied to decolorize methylene blue. The total dehydrogenase activity was independent of the sex, strain, and age of the animals, both normal and tumor-bearing. The addition of 0.5 mg. of coenzyme I markedly accelerated the total dehydrogenase activity of the muscle, liver, and brain of normal and tumor-bearing animals, the degree of acceleration in tissues from tumor-bearing animals being about the same as that in tissues from normal animals. The addition of the same amount of coenzyme I to extracts of the different tumors resulted in a pronounced stimulation of activity. The total dehydrogenase activity of the spleen from normal and tumor-bearing animals was not accelerated to any appreciable extent by the addition of a large amount of coenzyme I.

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Dehydrogenase Studies of Tissue from Normal and Tumor-bearing Mice

II. Lactic and Malic Dehydrogenases

Sr. M. PETRA LENTA, O.S.B., AND Sr. M. AGATHA RIEHL, O.S.B.

(From the College of St. Scholastica, Unit of the Institutum Diti Thomae, Duluth 2, Minnesota)

A high concentration of lactic acid in rat hepatoma has been reported by Kishi, Fujiwara, and Nakahara (5), as compared with the content of normal liver. Undoubtedly, this accumulation of lactic acid in tumor tissues is associated with the high rate of glycolysis in malignant tissues; but, since so little lactate is removed by oxidative processes, an impairment of enzyme activity is suggested. Elliott, Benoy, and Baker (1) have reported that the ability of tumor tissue to oxidize lactate to pyruvate, and succinate to fumarate, appeared to be defective. These investigators also found that the formation of succinate from pyruvate seemed to proceed at a fair rate and that an equilibrium between fumarate and malate could occur in tumor tissue. Waterman (11) concluded from his experiments that lactic dehydrogenase was lacking in cancerous tissue; but Von Euler, Malmberg, and Günther (9) have reported that a dialyzed extract of Jensen rat sarcoma rapidly oxidized lactic and malic acids. Von Euler, Adler, and Günther (8) further observed that the lactic and malic dehydrogenase systems of the Jensen rat sarcoma were not appreciably different from those of muscle. Von Euler, Malmberg, Günther, and Nystrom (10), however, considered that, while lactic acid and other hydrogen donors were present in excess in Jensen sarcoma, the deficient oxidative mechanism was associated with a lowered activity of the flavin enzymes and coenzymes. More recently, Potter (6) has investigated the malic dehydrogenase activity of normal rat liver and rat hepatoma. In the complete malic dehydrogenase system, the oxygen uptake of hepatoma was found to be much lower than that of normal liver.

The present study was designed to analyze the comparative lactic and malic dehydrogenase activities of various organs of normal and tumor-bearing mice.

EXPERIMENTAL

Of the animals used, 8 were normal C57, 35 were dba, and 31 were Rockland albino mice of both sexes and different ages. The dbrB adenocarcinoma was carried in dba mice, and the sarcoma 180 in the Rockland mice; at the time of experiment the tumors ranged between 8 and 25 mm. in diameter. All animals were maintained on a standard Rockland mouse-pellet diet. In preparing the tumor tissue for analysis, the necrotic and hemorrhagic areas were carefully removed.

The lactic and malic dehydrogenases were prepared according to the method of Green (2) with modifications. Essentially, the procedure consisted in the preparation of a dialyzed extract of enzymes obtained from the tissues as an acetone powder. According to the method of Green (2), the original tissue was allowed to extract for $\frac{1}{2}$ hour with ice water before precipitation with acetone. In the present study this extraction period was omitted because the amount of tissue rarely exceeded 1.5 gm. wet weight. The entire mixture was precipitated with acetone without previously pressing it through muslin, as prescribed by Green's method.

Immediately upon decapitation of the animals the organs to be analyzed were weighed and then ground thoroughly with a very small quantity of purified sand and a few milliliters of cold distilled water. The resulting thin paste was then poured into 3 volumes of cold acetone. The precipitate thus formed was filtered on a very small Büchner funnel and then washed with acetone and ethyl ether. This dried acetone powder was freed of the last traces of ether and then rubbed up with a small volume of cold distilled water, 2.0 ml. of water being used for every 0.1 gm. (wet weight) of tissue. The suspension was transferred quantitatively to Cellophane bags and dialyzed overnight against distilled water at 0°C. The preparation was then centrifuged at 800 r.p.m. for about 5 min-

utes and the precipitate discarded. The supernatant, which contained both lactic and malic dehydrogenase, was removed immediately and kept at 0° C. when not in use.

The dehydrogenase activity of the tissue preparations was measured according to the Thunberg technic. To the Thunberg tube proper were added 0.25 ml. methylene blue 1:5000; 1.0 ml. phosphate buffer, pH 7.2; 0.5 ml. of a 0.1 M substrate (sodium lactate or sodium malate); 0.2 ml. of a 2 M potassium cyanide adjusted to pH 8.0; and 0.5 mg. of a coenzyme I solution having a concentration of about 1 mg. per milliliter. Two ml. of the chilled enzyme solution were placed in the hollow stopper of the Thunberg tube.

Coenzyme I was prepared according to the method of Williamson and Green (12). Although the percentage purity was not determined, successive batches were standardized against one another. The strong potassium cyanide solution was added to fix the products formed in the dehydrogenase reaction. The evacuation of the tubes was accomplished with a high-vacuum pump at about 12 mm. for 5 minutes and was facilitated by gentle agitation of the tubes during this time. The tubes were then placed in a constant-temperature water bath at 37° C. for 10 minutes. At the end of this equilibration period the enzyme-containing solution was allowed to react with the substrate, and the time required for decolorization of the methylene blue was determined. Tests were made in duplicate. The dehydrogenase activity was expressed at minutes⁻¹ × 10².

RESULTS

The values obtained for the malic and lactic dehydrogenases in various organs from normal and tumor-bearing mice, as well as in the dbrB adenocarcinoma and sarcoma 180 transplanted tumors are given in Tables 1-6, in which the results of 924 determinations are reported. Eighty-nine per cent of the duplicate determinations checked within 2 minutes, the remainder varying not more than 4 minutes. There was a variation of ± 3 minutes in the enzyme activity of analogous organs from animals of the same strain. The rate of decolorization in the presence of tumor tissue varied within a maximum of ± 5 minutes, with two-thirds varying less than ± 2 minutes.

In the case of the lactic dehydrogenase activity of the spleen of both normal and tumor-bearing animals, complete decolorization in 72 per cent of the 33 determinations did not take place within 2 to 3 hours. There was, however, a change from the original deep-blue color to a green within the first half-hour of that time, but further decolorization

was not appreciable after that period. In the remaining 9 instances (28 per cent), complete decolorization occurred within 20 to 60 minutes. This variation in activity may be associated with the hemopoietic nature of the organ and the amount of blood present.

DISCUSSION

While the presence of tumors has been associated with the lowering of certain enzyme activities in organs remote from the site of the neoplasm and not invaded by the tumor (4), in the experiments reported herein neither the lactic nor the malic dehydrogenase activity of organs of animals bearing transplanted tumors appears to be influenced by the growing neoplasm. The values were not altered significantly even when the tumors had attained a very large size. Furthermore, this activity was independent of the strain, sex, and age of the animal.

The lowest values for the lactic and malic dehydrogenases were obtained in the dbrB adenocarcinoma and in the sarcoma 180. This would, perhaps, indicate a less efficient oxidative mechanism in tumor tissues. The value for the malic dehydrogenase, however, lies within the lowest range of those for normal tissues analyzed, viz., the spleen. This finding is in keeping with the observation of Greenstein (3) that the activity of the catalytic systems in tumors generally stands in the mid-region or near the lower extreme of the range of enzyme activities of normal tissues.

It is also significant that the enzyme preparations from the spleen of both normal and tumor-bearing animals failed in 72 per cent of the experiments to decolorize methylene blue completely when sodium lactate was the substrate. This phenomenon can probably be attributed to the low concentration of lactic dehydrogenase in this tissue, but the possibility of enzyme inhibitors cannot be excluded. A low concentration of enzyme systems requiring coenzyme I is indicated, however, since the addition of large quantities of coenzyme I failed to cause an appreciable acceleration of the rate at which aqueous extracts of spleen decolorized methylene blue (7). The fact that the values for the activity of malic dehydrogenase—another coenzyme I-requiring system—are lower for the spleen than for any of the other normal tissues analyzed further substantiates the supposition that certain enzymes requiring coenzyme I are present in small quantities in the spleen. Thus the failure of spleen extracts to decolorize methylene blue when lactate is the substrate can probably be ascribed to a low concentration of lactic dehydrogenase in this organ rather than to the presence of inhibitors of the enzyme system.

TABLE 1

DECOLORIZATION TIME OF METHYLENE BLUE BY LACTIC AND MALIC DEHYDROGENASE IN SKELETAL MUSCLE OF NORMAL AND TUMOR-BEARING MICE

STRAIN OF MICE	DEHYDROGENASE	NO. DETERMINATIONS	MEAN	DECOLORIZATION TIME (MIN.)	S.D.	VELOCITY (MIN. ⁻¹ × 10 ²)
NORMAL ANIMALS—MUSCLE						
dba	{Lactic	14	5.8	(4.0-9.0)	±1.55	17.2
	{Malic	18	4.7	(2.0-9.0)	±2.39	21.2
Rockland	{Lactic	12	4.1	(3.25-5.0)	±1.65	24.4
	{Malic	12	2.5	(1.0-8.0)	±2.50	40.0
C 57	{Lactic	8	5.3	(4.5-9.0)	±0.65	18.8
	{Malic	4	3.3	(3.0-3.5)	±0.24	30.3
TUMOR-BEARING ANIMALS—MUSCLE						
dbrB in dba	{Lactic	20	6.5	(4.0-9.0)	±1.75	15.3
	{Malic	30	4.5	(1.0-8.0)	±1.74	22.2
S 180 in Rockland	{Lactic	14	4.8	(4.0-6.0)	±0.78	20.8
	{Malic	14	2.6	(1.0-5.0)	±1.37	38.4

TABLE 2

DECOLORIZATION TIME OF METHYLENE BLUE BY LACTIC AND MALIC DEHYDROGENASE IN THE SPLEEN OF NORMAL AND TUMOR-BEARING MICE

STRAIN OF MICE	DEHYDROGENASE	NO. DETERMINATIONS	MEAN	DECOLORIZATION TIME (MIN.)	S.D.	VELOCITY (MIN. ⁻¹ × 10 ²)
NORMAL ANIMALS—SPLEEN						
dba	{Lactic	16	>2 hours in 12 determinations; 60' and 20'			
	{Malic	14	11.0 (7-17)	±3.34		9.1
Rockland	{Lactic	14	>2 hours in 10 determinations; 50' and 30'			
	{Malic	10	10.6 (7-18)	±3.83		9.4
C57	{Lactic	8	>2 hours in 6 determinations; 22'			
	{Malic	8	9.7 (4-12)	±3.34		10.3
TUMOR-BEARING ANIMALS—SPLEEN						
dbrB in dba	{Lactic	18	>2 hours in 12 determinations; 30', 22', and 25'			
	{Malic	22	9.5 (5-15)	±2.87		10.5
S 180 in Rockland	{Lactic	10	>4 hours in 8 determinations; 30'			
	{Malic	8	12.5 (11-14)	±1.12		8.0

TABLE 3

DECOLORIZATION TIME OF METHYLENE BLUE BY LACTIC AND MALIC DEHYDROGENASE IN KIDNEY OF NORMAL AND TUMOR-BEARING MICE

STRAIN OF MICE	DEHYDROGENASE	NO. DETERMINATIONS	MEAN	DECOLORIZATION TIME (MIN.)	S.D.	VELOCITY (MIN. ⁻¹ × 10 ²)
NORMAL ANIMALS—KIDNEY						
dba	{Lactic	22	4.0	(2.0-6.0)	±1.41	25.0
	{Malic	26	2.2	(1.0-5.0)	±1.19	45.4
Rockland	{Lactic	12	4.1	(3.0-6.0)	±1.34	24.4
	{Malic	10	2.3	(1.5-3.5)	±0.68	43.5
C57	{Lactic	12	4.0	(2.0-6.0)	±1.29	25.0
	{Malic	10	2.6	(1.0-4.0)	±1.02	38.4
TUMOR-BEARING ANIMALS—KIDNEY						
dbrB in dba	{Lactic	22	4.6	(2.0-7.0)	±1.91	21.7
	{Malic	22	1.7	(1.0-3.0)	±0.81	58.8
S 180 in Rockland	{Lactic	16	6.1	(3.0-10.0)	±2.03	16.4
	{Malic	22	2.6	(1.0-6.0)	±1.48	38.4

TABLE 4

DECOLORIZATION TIME OF METHYLENE BLUE BY LACTIC AND MALIC DEHYDROGENASE IN LIVER OF NORMAL AND TUMOR-BEARING MICE

STRAIN OF MICE	DEHYDROGENASE	No. DETERMINATIONS	MEAN	DECOLORIZATION TIME (MIN.) Range	S.D.	VELOCITY (MIN. ⁻¹ × 10 ²)
NORMAL ANIMALS—LIVER						
dba	{Lactic	18	4.9	(2.0–11.0)	±2.75	20.4
	{Malic	28	2.4	(1.0–4.0)	±0.94	41.6
Rockland	{Lactic	24	4.3	(3.0–10.0)	±1.99	23.2
	{Malic	22	1.5	(1.0–4.0)	±0.92	66.6
C57	{Lactic	20	5.6	(3.5–8.0)	±1.61	17.8
	{Malic	10	2.4	(1.5–6.0)	±1.70	41.6
TUMOR-BEARING ANIMALS—LIVER						
dbrB in dba	{Lactic	32	5.8	(2.0–11.0)	±2.29	17.2
	{Malic	30	2.2	(1.5–4.0)	±0.71	45.4
S 180 in Rockland	{Lactic	16	6.5	(3.5–10.0)	±2.46	15.3
	{Malic	30	2.6	(1.0–4.0)	±0.80	38.4

TABLE 5

DECOLORIZATION TIME OF METHYLENE BLUE BY LACTIC AND MALIC DEHYDROGENASE IN BRAIN OF NORMAL AND TUMOR-BEARING MICE

STRAIN OF MICE	DEHYDROGENASE	No. DETERMINATIONS	MEAN	DECOLORIZATION TIME (MIN.) Range	S.D.	VELOCITY (MIN. ⁻¹ × 10 ²)
NORMAL ANIMALS—BRAIN						
dba	{Lactic	14	7.8	(3.0–13.0)	±3.13	12.8
	{Malic	16	3.7	(2.0–8.0)	±1.86	27.0
Rockland	{Lactic	14	7.3	(4.5–10.0)	±2.01	13.7
	{Malic	16	3.5	(1.0–9.0)	±2.91	28.5
C57	{Lactic	4	7.0	(6.0–8.0)	±1.00	14.3
	{Malic	4	2.0	(2.0)	±0.00	50.0
TUMOR-BEARING ANIMALS—BRAIN						
dbrB in dba	{Lactic	26	9.9	(5.0–14.0)	±2.87	10.1
	{Malic	30	3.1	(1.0–7.0)	±1.74	32.3
S 180 in Rockland	{Lactic	12	10.6	(6.0–16.0)	±3.99	9.4
	{Malic	16	3.4	(2.0–6.0)	±1.64	29.4

TABLE 6

DECOLORIZATION TIME OF METHYLENE BLUE BY LACTIC AND MALIC DEHYDROGENASE IN TUMOR TISSUE

STRAIN OF MICE	DEHYDROGENASE	No. DETERMINATIONS	MEAN	DECOLORIZATION TIME (MIN.) Range	S.D.	VELOC. TY (MIN. ⁻¹ × 10 ²)
dbrB in dba	{Lactic	32	17.3	(12–22)	±3.38	5.7
	{Malic	22	9.6	(5–12)	±2.92	10.4
S 180 in Rockland	{Lactic	18	19.5	(15–24)	±2.79	5.1
	{Malic	22	13.7	(7–20)	±4.31	7.3

SUMMARY

Studies of the comparative activities of lactic and malic dehydrogenases of the liver, kidney, brain, muscle, and spleen of various strains of mice revealed these enzyme activities to be independent of the strain, sex, and age of the animal. The presence of a transplanted tumor did not influence the activity of these enzymes in any of the organs analyzed. The values for lactic and malic dehydrogenases of a transplanted adenocarcinoma and sarcoma were lower than those of any non-neoplastic tissue studied, with the exception of the malic dehydrogenase of spleen from both normal and tumor-bearing mice. The malic dehydrogenase activity of the spleen corresponded closely to that of the same enzyme in both the *dbrB* adenocarcinoma and sarcoma 180. With lactate as substrate, enzyme preparations from the spleen of both normal and tumor-bearing mice failed to decolorize methylene blue completely in most cases. This can probably be attributed to the low concentration of lactic dehydrogenase in that organ.

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Serial Intraocular Transplantation of Frog Carcinoma for Fourteen Generations*

HANS SCHLUMBERGER, M.D., † AND BALDUIN LUCKÉ, M.D.

(From the Laboratory of Pathology, School of Medicine, University of Pennsylvania
Philadelphia 4, Pennsylvania)

The adenocarcinoma which commonly affects the kidneys of leopard frogs (*Rana pipiens*) may readily be transplanted to the anterior chamber of the eye (1, 2). Here the rate and manner of its growth can be observed directly through the thin, transparent cornea with the microscope, and permanent objective records can be obtained by photographs. Our previous studies have been based largely upon intraocular transplants during the first generation, i.e., transplants from primary tumors in the kidney. The present study deals with the behavior of tumors that became established in the eye and were then transplanted serially. The experiments are concerned with several interrelated questions: Is it possible to transplant the frog carcinoma for many generations and thus maintain it over a considerable period? If so, what effect will repeated transfer and prolonged maintenance in the anterior chamber have upon the tumor—upon its rate of growth; its manner of growth; its malignant properties, such as invasiveness; its response to changes in temperature; and its behavior when transplanted to alien species? Finally, how will a metastatic carcinoma behave in serial transplantation compared to a primary tumor?

MATERIALS AND METHODS

Details of the method for intraocular transplantation have been described in a previous publication (2). Briefly stated, small portions of primary tumor, 1 to 2 mm. in size, were planted in the anterior chamber of the eyes of frogs with a finely pointed forceps through a small incision at the sclerocorneal junction. Aseptic precautions were observed, and care was taken to have the bits of tumor of approximately uniform size. The subsequent fate of the transplants was then observed at intervals of a few days through the slit-lamp microscope or a modification of the instrument. Permanent records were obtained by photographs.

* This study was aided by a grant from the Donner Foundation.

† Now at Ohio State University, Columbus, Ohio.

After the transplants had grown to fill the anterior chamber, the animals were anesthetized with ether, the cornea was cut away, the tumor removed and subdivided and reinoculated into the eyes of other frogs. The behavior of the new generation of tumors was then studied by the means outlined above.

In the main group of experiments the material for the first generation of transplants came from a large bilateral kidney tumor. The frogs used were unselected adults. They were kept separately in small aquarium jars in a moist atmosphere at a constant temperature of 28° C.

Details of the experiments as regards temperature, heterotransplantation, and transplantation of metastatic tumors will be given in the pertinent sections.

RESULTS

Incidence of takes and rate of growth in successive generations of transplanted tumors.—In the first generation the tumors grew in only 1 of the 10 frogs inoculated. When the tumor in this frog had filled the anterior chamber and bits of it had been retransplanted, 4 out of 5 transplants became established; in the next generation 6 out of 7 grew. After the third generation the incidence of takes rose to 100 per cent and was maintained at this level for the remainder of the experiment, i.e., from the fourth to the fourteenth generation (Table 1). The experiment was then discontinued, since it had shown that the kidney carcinoma of the frog can successfully be maintained in the anterior chamber for a considerable number of generations and over a period of 2 years and 3 months.

The form and size of all the tumors in successive generations were compared by means of photographs taken periodically, usually at weekly intervals. A representative group of such records is given in Figs. 1 to 6. The figures illustrate the pattern of growth which is characteristic of this tumor. In the early stages the transplants grew slowly; but, once they became established and vascularized, the rate was accelerated, and the

tumors enlarged rapidly. The final stage of growth is not shown in the photographs; as the mass filled the anterior chamber, increase in size became slower and slower.

The rate of vascularization and the size attained by the implants remained similar in all generations. Thus tumors in the fourteenth generation established vascular connections as quickly and attained as great a size as in earlier generations (Figs. 7 to 9).

The photographic records served as one means for comparing rates of growth in different generations. Another means for such comparison was the time required for the majority of transplants to fill the anterior chamber. Inspection of the last column in Table 1 shows that these times varied widely, from 37 to 100 days. There was no trend toward either a longer or a shorter rate in successive generations. Since, as stated above, care was taken to make transplantation as uniform a procedure as possible, the variations in growth rates must depend on unknown factors.

TABLE 1

SERIAL INTRAOCCULAR TRANSPLANTATION OF FROG CARCINOMA THROUGH FOURTEEN GENERATIONS*

Generation of tumor	No. of animals inoculated	No. of takes	Average time for transplants to fill anterior chamber (days)	Average time for replanted tumors to fill anterior chamber (days)*
			VI Rapid rate	
I	10	1	60	57
II	5	4	88	60
III	7	6	51	
IV	10	10	38	
V	7	7	48	
VI	13	13	43	
VII	14	14	60	
VIII	11	11	46	
IX	25	25	100	
X	10	10	43	
XI	23	23	79	
XII	6	6	71	
XIII	11	11	58	
XIV	14	14	69	
Total	166	..	854	

* The table shows for each generation of transplanted tumors the number after the third generation. The growth rates, however, did not become uniform but varied widely.

In confirmation of the view that serial transplantation did not effect a sustained change in rate of growth is a group of experiments which are summarized in Table 2. In several generations there were encountered transplants which had grown decidedly more rapidly or more slowly than their fellows. Thus, during the sixth generation in which the majority of the tumors required 43 days to fill the anterior chamber, one grew so rapidly as to require but approximately half this time, 22 days. But when transplants from this tumor were inoculated, the previous rapid rate was not main-

tained, the rate of growth approximating that of the controls, i.e., of the majority of tumors of the seventh generation (cf. first pair of figures in Table 2). Other and similar examples are given in the table, both for rapidly and for slowly growing tumors. Thus it may be concluded that no permanent alteration in rate of growth was developed by selective transplantation.

Manner of growth of transplants in successive generations.—Previous studies showed that the car-

TABLE 2

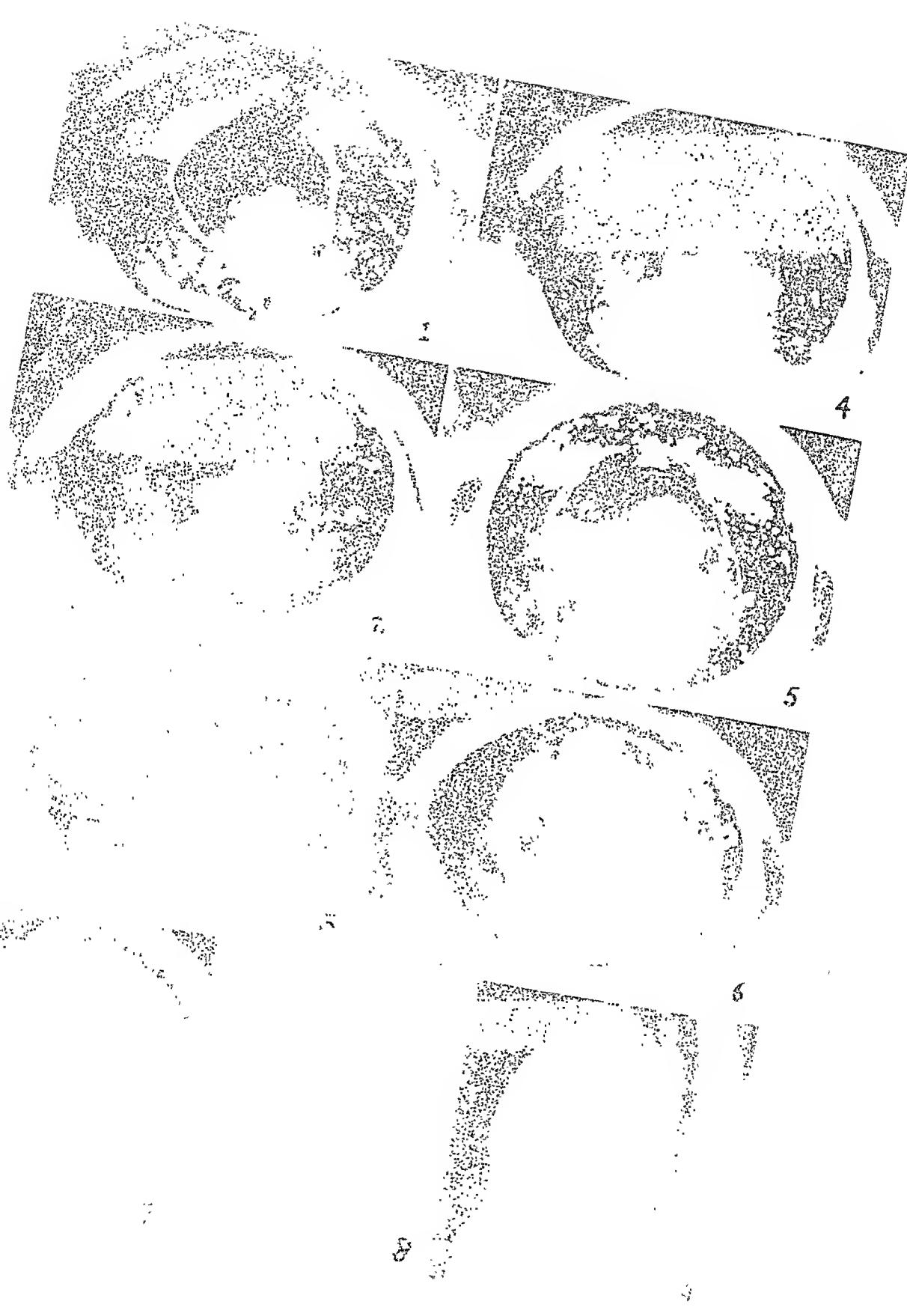
TIME REQUIRED TO FILL ANTERIOR CHAMBER UPON RE-TRANSPLANTATION OF STRAINS THAT PREVIOUSLY HAD DIFFERED FROM THE MAJORITY BY EITHER RAPID OR SLOW GROWTH

Designation of strain of tumor transplant	Time required by tumor to fill anterior chamber (days)	No. of animals subsequently inoculated	Average time for replanted tumors to fill anterior chamber (days)*
VI Rapid rate	22	6	57
VI Average rate	43	14	60
VII Rapid rate	38	6	64
VII Average rate	60	11	46
XI Rapid rate	47	9	71
XI Average rate	79	6	65
VIII Slow rate	103	6	50
VIII Average rate	60	11	46
VIII Slow rate	98	3	59
VIII Average rate	46	25	90

* Comparison of the pairs of figures in this column shows no significant differences in growth rates, i.e., no permanent alteration by selective transplantation has been effected.

cina when transplanted to the anterior chamber of the eye develops according to definite structural patterns, their type depending on the immediate physical environment (2). Three such morphogenetic patterns were recognized: (a) Where the tumor grew out into the aqueous humor, uninhibited by solid tissue, it formed cylinders, tubules, cysts, or papillary structures, which gradually lengthened and became more complex by branching. (b) Where the growing tips or margins of the outgrowths made contact with a firm surface, such as lens or cornea, adhesion took place and a change in form occurred; at this point the proliferating cells spread as flat membranes over the supporting surface. (c) Where contact was made with a loose, distensible tissue, such as iris, an invasive acinar type of growth resulted.

We have as yet encountered no factors which bring about a decisive change in this unvarying pattern. But, as stated above, our observations hitherto were usually made on tumor transplants in the first generation. Would serial transfer, and hence long residence in a new environment—the



aqueous humor—modify the growth pattern? The experiments demonstrated clearly that no changes in pattern developed. The tumors in all fourteen generations grew in a manner quite similar to that observed previously (3). Illustrative examples are to be seen in Figs. 1 to 6.

Development of corneal erosion.—We now come to a conspicuous effect of serial transplantation, i.e., increase in the ability of the tumor transplants to invade and destroy adjacent tissue. Although the frog carcinoma in its normal habitat, the kidney, is an invasive tumor which tends to destroy the surrounding renal tissue, its transplants to the eye, during the first few generations at least, very rarely invaded the ocular tissues other than the loose and spongy iris. The hard, unyielding cornea, on the contrary, usually offered an effective barrier to the spread of the cancer. Upon repeated transfer, however, and hence prolonged adaptation of the transplant to conditions prevailing in the eye, the tumor reacquired its invasive properties. Using perforations of the cornea as a readily visible criterion of invasion and subsequent destruction, it was found that such perforations occurred only irregularly and in relatively few animals during the earlier generations of transplants, whereas the incidence rose greatly in the later generations. Thus in the first seven generations of transplants, perforations of the cornea took place in only three, whereas in the following seven generations it occurred in all. The percentage incidence during the first seven generations was 12.6; it rose to nearly four times that rate, 44.8, during the following seven generations (Table 3).

The appearance and development of a corneal perforation is shown in Figs. 10 to 12. Fig. 10 illustrates a tubulomembranous growth in the eighth generation; it is adherent to the inner surface of the cornea, occludes the pupil, and has caused a slight outward bulging of the cornea. Careful examination with high magnification by the slit-lamp microscope, before and after instillation of fluorescein (an aid in making injuries to the outer

surface visible), failed to reveal any abrasion of the corneal surface. During the following 17 days the lesion changed very little; but a second, larger, and complete perforation developed near by, through which portions of the tumor protruded (Fig. 11). During the following 3 weeks the eroded area rapidly increased in size; it had a punched-out appearance, and the protruding tumor gradually sloughed off (Fig. 12). In the example given, only one complete erosion occurred, even though another area of corneal bulging was present. Occasionally, two separate corneal erosions were encountered (Fig. 19).

TABLE 3

INCIDENCE OF CORNEAL PERFORATION IN SUCCESSIVE GENERATIONS OF INTERAOCCULAR TRANSPLANTS OF FROG CARCINOMA*

Generation no.	No. of animals living over 21 days	No. of animals with corneal perforation	Percentage with corneal perforation
I	10	0	0
II	5	0	0
III	7	2	28.5
IV	7	2	28.5
V	5	0	0
VI	10	0	0
VII	14	3	21.4
VIII	6	1	16.6
IX	17	7	41.2
X	10	8	80.0
XI	23	10	43.5
XII	6	2	33.3
XIII	11	5	45.5
XIV	14	6	42.9

* Corneal perforation occurred irregularly and in relatively few animals during earlier generations of transplants. After the eighth generation the incidence rose greatly.

Once an erosion had developed, the tumor usually protruded through the opening. As a rule, the mass sloughed off, through injury or infection. But sometimes tumors that were well vascularized continued to grow for weeks, even though they were exposed; in such cases the nictitating membrane had served as a protecting envelope (Figs. 16 to 18).

Figs. 1 to 9.—All figures are unretouched photographs of living tumors. All magnifications are at $\times 9$, excepting Fig. 8, which is at $\times 20$.

Figs. 1 to 6.—Rate of growth of representative transplants in the eighth generation. The photographs were taken 12, 19, 26, 33, 40, and 51 days, respectively, after transplantation.

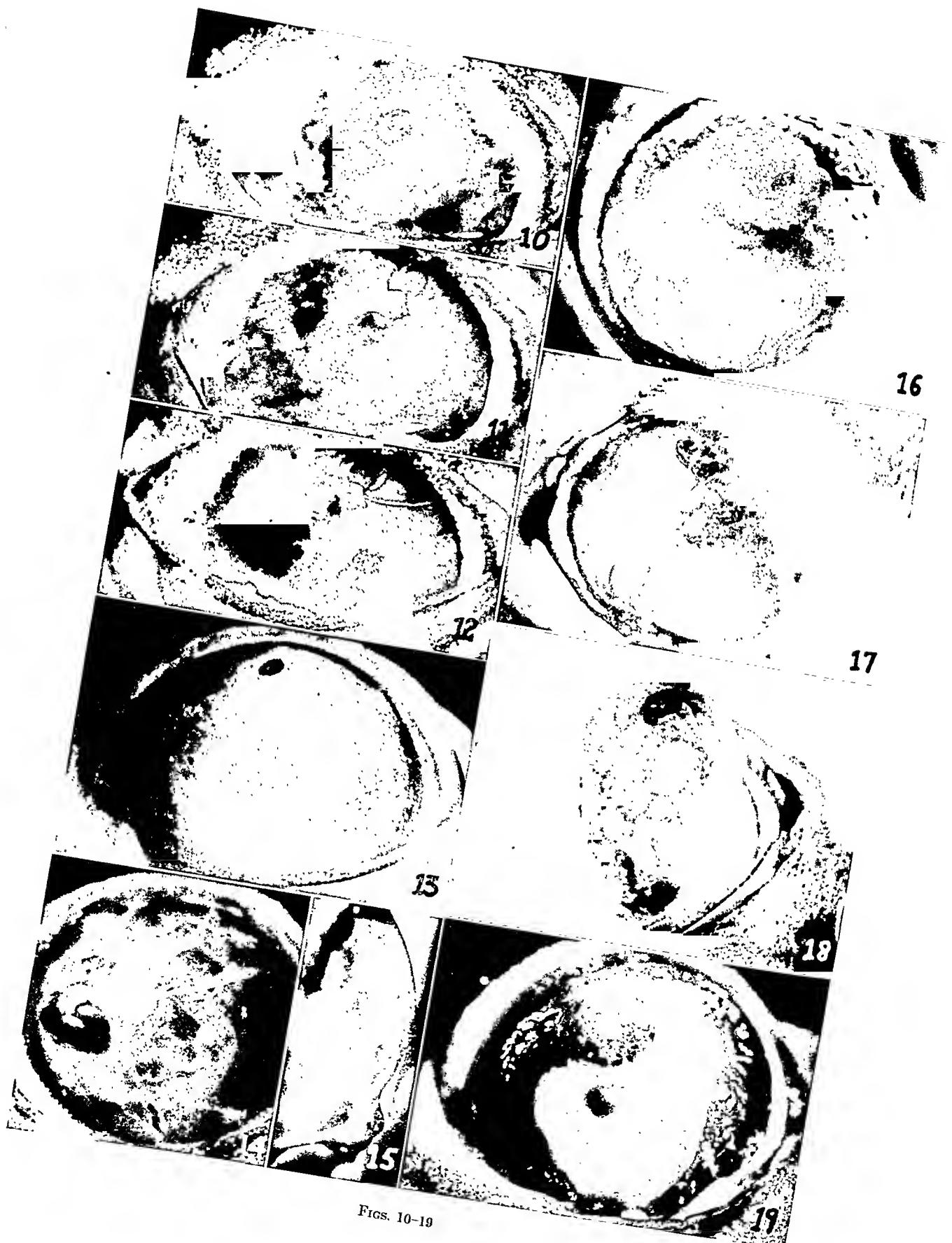
Figs. 1 and 2.—During the earlier stages the outgrowths were tubules which rapidly became distended with fluid, thus forming small cysts.

Figs. 3 and 4.—Through thickening of their walls and budding inward of new tubules, the cysts gradually became transformed into solid masses.

Figs. 4 and 5.—Contact of the growth with lens resulted in the formation of a membranous carpet of tumor cells.

Fig. 6.—Within 11 days after last photograph was taken the now well-vascularized tumor had almost filled the anterior chamber.

Figs. 7 to 9.—A tumor in the fourteenth generation, 69 days after transplantation. Fig. 7 gives a front view, Fig. 9 a side view, of the growth. In both photographs note the vessels which arise at the sclerocorneal junction and pass into the tumor. Fig. 8 is a photograph of these vessels at higher magnification. By careful focusing of the microscope and observation of the blood flow, it could easily be seen that the vessels penetrated into the tumor tissue.



FIGS. 10-19

As in the destruction of any tissue by a neoplasm, the mechanism of corneal erosion is complex and not completely understood. A constant factor is adhesion of an actively growing tumor to the inner surface of the cornea. A factor of importance, no doubt, is invasion of this structure by tumor cells which insinuate themselves into the interstices, thereby interfering with nutrition and weakening this normally tough tissue. Also, increased intraocular tension due to growth of tumor must play a part; it manifests itself especially in the areas of lessened resistance, where it leads to localized bulging (Figs. 14 and 15). Erosion, however, may sometimes develop without previous local bulging; in such cases the area to which the intraocular tumor is adherent rapidly becomes punched out as if the cells had undergone lysis (Fig. 13).

In brief, these experiments indicate that one of the malignant properties, i.e., invasiveness, of intraocular transplants of the frog carcinoma is enhanced by repeated transfer and prolonged maintenance in the anterior chamber of the eye.

The effect of temperature on growth of transplants.—The frog carcinoma is particularly good material for studying the effects of temperature on growth of neoplasms. Since the temperature of the frog is practically that of its surroundings, we have an opportunity to investigate the effects of temperature over a far wider range than is possible in warm-blooded animals. Previously we reported experiments in which the effect of temperature was studied by direct microscopic examination of living intraocular transplants mainly in the first generation, with a few, however, observed during four generations (3). It was found that at a high temperature, 28° C., the rate of growth was much accelerated, that the tumors became more rapidly vascularized, and that the tubular outgrowth tended to become cystic through accumulation of

fluid. At a low temperature, 7° C., growth was greatly retarded but did not cease entirely; vascularization was poor, the outgrowth tended to be short and stubby, and rarely became cystic.

In our present experiments, all the tumor-bearing frogs were kept at a constant temperature of 28° C. It seemed of interest to find out whether prolonged sojourn at this abnormally high temperature had so altered the neoplastic tissue that it could no longer survive at a low temperature. Accordingly, when the tumors had been growing at 28° C. for 94 weeks and were in the twelfth generation, the frogs were placed in a constant-temperature room at 7°, where they were kept for 4 weeks. During this time the transplants remained practically unchanged in size; there was little or no evidence of growth. But the exposure to this low temperature had not harmed the tumors; for, when the frogs were returned to 28°, outgrowths became apparent within 3 days and continued without interruption in a manner entirely similar to the growth in the control group, which had remained at high temperature. A group of representative photographs are given in Figs. 20 to 23.

The experiments indicate that growth of intraocular transplants is not altered either quantitatively or qualitatively by long exposure to a high temperature.

The effect of serial transfer on subsequent heterotransplantation.—From earlier experiments we had learned that the kidney carcinoma of the leopard frog may as readily be established in the eyes of other species of the same family of frogs as in the natural host. In a species from a different family (the toad) the proportion of successful transplantations was somewhat less; but in animals of different classes of cold-blooded vertebrates the transplants regressed (4). It seemed quite possible that, after 2 years of growth in the anterior chamber, the tumor might be more adapted to the

Figs. 10 to 19.—All figures are unretouched photographs of living tumors. Mag. $\times 9$. The photographs show the development and appearance of corneal perforations.

Figs. 10 to 12.—In Fig. 10 is shown a flat tubulomembranous growth from a transplant in the eighth generation. The tumor is adherent to the central portion of the cornea, which is bulging outward. Fig. 11 shows the appearance 17 days afterward; the bulge has now become eroded. A second, larger erosion has developed near by, through which parts of the tumor are protruding. By close inspection of the photographs it may be seen that numerous small vessels extend from the sclerocorneal junction to the edge of the larger erosion. Fig. 12 shows the eroded area as it appears 3 weeks after the preceding photograph was taken. The second erosion has become much larger. Note the abundant vessels supplying the tumor.

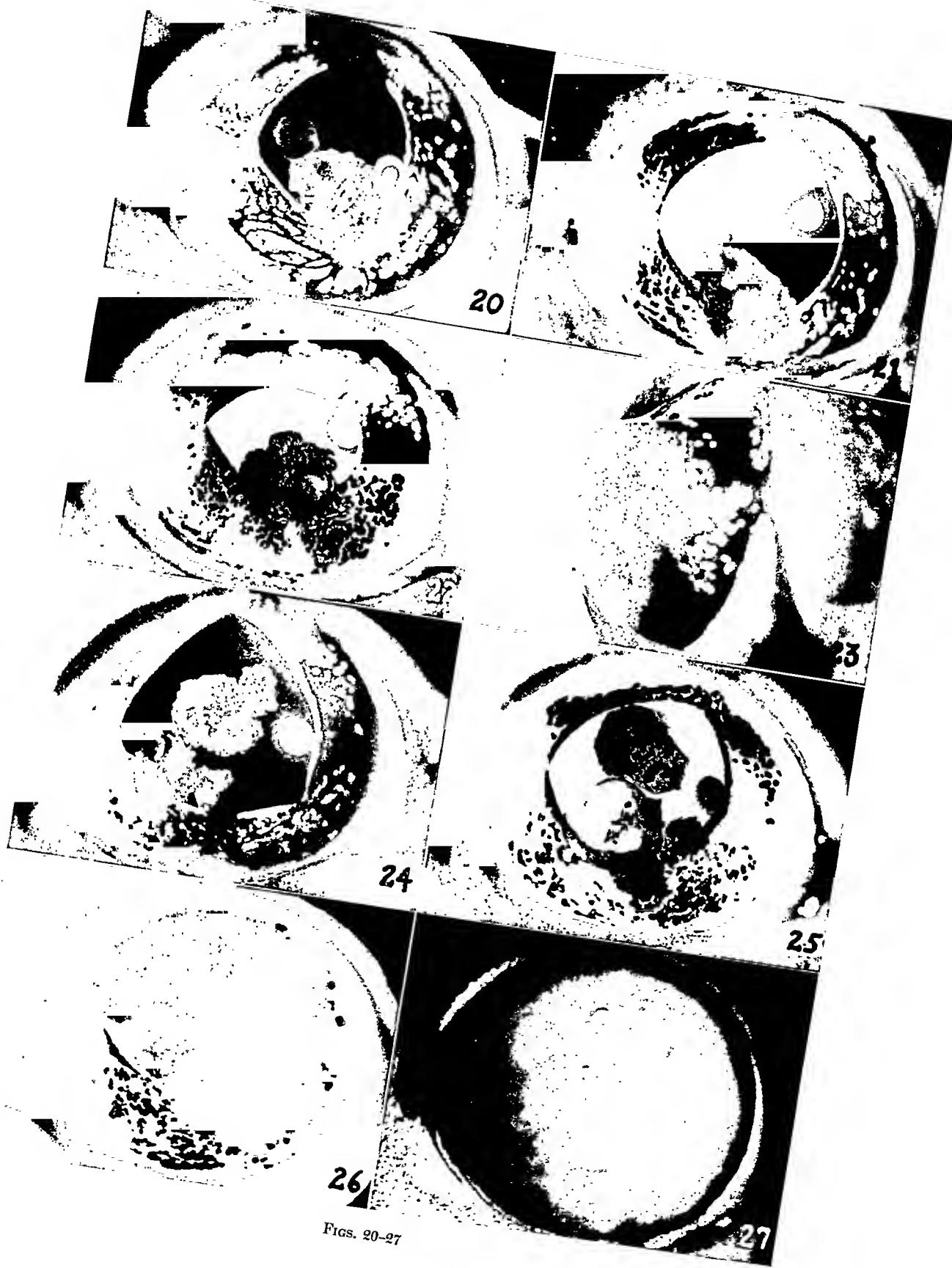
FIG. 13.—This photograph of a transplant in the twelfth generation shows a sharply circumscribed area of corneal destruction which is not accompanied by protrusion of the tumor.

Figs. 14 and 15.—Front and side views of a corneal perforation through which the tumor protrudes. Note that the normal curvature of the cornea is retained except at the point of erosion. (From a transplant in the tenth generation.)

Figs. 16 to 18.—In Fig. 16 is shown the appearance of a tumor in the tenth generation, 20 days after inoculation. Note the excellent vascularization. Eleven days later (Fig. 17) the entire anterior chamber is filled with tumor, and a large centrally located corneal perforation has developed, through which the mass protrudes. During the next 11 days almost the entire cornea has been destroyed.

FIG. 18.—The protruding tumor has been protected from injury by the nictitating membrane (which in the photograph has been pulled away).

FIG. 19.—This tumor in the seventh generation occupies only a portion of the anterior chamber, but two perforations have developed.



FIGS. 20-27

aqueous humor of alien species. However, this did not prove to be the case. When in the thirteenth generation the neoplasm was transferred to the eyes of 4 bullfrogs (*Rana catesbeiana*), 6 toads (*Bufo americanus*), and 3 goldfish (*Carassius auratus*), it grew in all the bullfrogs (causing erosion of the eye in 1); in the toads it became quickly established in 2 and grew more slowly in 2 others. In the goldfish the transplants were promptly surrounded by a cellular exudate and at no time showed growth. Thus, while serial transplantation had increased the number of takes in the natural host, it had not facilitated transplantation to alien hosts.

Serial transplantation of a metastatic tumor.—The formation of metastatic colonies by a cancer indicates that its malignant properties are fully developed; for its cells are now able to grow in tissues different from those of their origin (5). How would metastatic tumors behave when serially transplanted? Would they grow more luxuriantly in the anterior chamber of the eye than did primary tumors? The material for investigating these questions was a large kidney tumor which had formed numerous colonies in the liver. Some of these were transplanted into the anterior chamber of 9 frogs, each animal receiving an entire small metastatic nodule. Nine other frogs were similarly inoculated with bits of the primary tumor. The subsequent experimental procedures were identical with those described in the introduction. None of the transplants of the primary tumor became established and all soon regressed. On the contrary, 1 of the 9 metastatic nodules grew vigorously; upon retransplanting it to the second generation, 10 of 11 tumors became established; thereafter, all the transplants grew (Table 4). This incidence of takes is very similar to that obtained for the primary tumor, studies of which have been summarized in Table 1. The transplants of the metastatic tumors were maintained for 385 days, during which they were passed through five generations. Their pattern of growth was entirely similar to that of

transplants from primary tumors. There was, during early stages, a period of relatively slow growth, followed by a later period of rapid growth (Figs. 24 to 27). The characteristic formations of tubules, cysts, and membranes were all observed. The rate of growth tended to be somewhat slower than the rate reported in Table 1 for a primary tumor, but no significance can be attributed to such minor variations.

The invasiveness of the metastatic tumor was not greater than that of a primary neoplasm when transplanted to the eye. Corneal invasion and perforation occurred in only 2 of the 43 animals; this is in keeping with the low incidence during earlier generations of implants from primary tumors. The experiments were discontinued after the fifth generation because it was evident that transplants from metastatic tumors reacted in the same way as did primary tumors.

TABLE 4
SERIAL INTRAOCCULAR TRANSPLANTATION OF
METASTATIC CARCINOMA THROUGH FIVE
GENERATIONS*

Generation of tumor	No. of frogs inoculated	No. of takes	Average time for transplants to fill anterior chamber (days)
I	9	1	83
II	11	10	88
III	7	7	70
IV	7	7	74
V	9	9	70

* Except for a somewhat slower rate of growth, the metastatic carcinomas behave similarly to the primary tumor, studies of which are summarized in Table 1.

SUMMARY

A renal carcinoma of the leopard frog has been successfully maintained in the anterior chamber of the eye through fourteen serial transplantations over a period of 2 years and 3 months. The characteristics of the growth of the tumor were observed *in vivo* with the slit-lamp microscope. After

Figs. 20 to 27.—All figures are unretouched photographs of living tumor. Mag. $\times 9$.

Figs. 20 and 21.—The effect of low temperature on growth of tumor. Fig. 20 shows the appearance of a transplant in the twelfth generation on the day that the frog, previously kept in a thermostat room at $28^{\circ}\text{ C}.$, was transferred to a constant temperature of $7^{\circ}\text{ C}.$ After remaining at the low temperature for 4 weeks, the tumor is seen to be almost unchanged; there is very little evidence of growth (Fig. 21).

Figs. 22 and 23.—The effect of high temperature on tumor growth. These photographs are of transplants belonging to the same series as those of the two preceding figures. The animal, which had been left at $28^{\circ}\text{ C}.$, remained at this temperature for 4 additional weeks. In Fig. 22 is given the appearance of the

transplant at the beginning of the experiment; in Fig. 23 at the end, the anterior chamber has almost been filled by the vigorous growth. Figs. 22 and 23 should be compared with Figs. 20 and 21, respectively, to show the growth-accelerating effect of temperature.

Figs. 24 to 27.—Growth of a liver metastasis after five serial transplantations in the eye. Fig. 24 shows the appearance of the tumor 1 week after inoculation; Fig. 25, the appearance 1 week later. In Fig. 26, taken 11 weeks after inoculation, about two-thirds of the anterior chamber has become filled; 1 week afterward the entire chamber has been occupied (Fig. 27). Compare the relatively slow rate during the first 2 weeks of the experiment with the rapid rate of growth during the last week.

repeated transfer, the growth of the implants became more invasive, as indicated by increasing frequency of corneal erosion. The general pattern of growth, however, as well as the response to temperature and heterotransplantation remained remarkably stable.

Metastatic tumors located in the liver were similarly studied in five generations. Both in vigor and in pattern the growth of these transplants was found to correspond closely to that of primary tumors. These results suggest that primary tumors and their metastases will grow in like manner when placed in a similar environment.

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Transplantation of Heterologous Tumors by the Intravenous Inoculation of the Chick Embryo^{*†‡}

DORIS H. BENDER, M.D., CHARLES E. FRIEDGOOD, M.D.,
AND HENRY F. LEE, M.D.

(From the Harrison Department of Surgical Research, School of Medicine, University of Pennsylvania
and the Children's Hospital of Philadelphia)

This report is concerned with the transplantation of cells of certain mammalian neoplasms into the living chick embryo by injection of these cells into the allantoic vein. Although the technic of allantoic vein injection has been reported previously, it has not been used heretofore as a technic for implanting tumor cells from the same species or from another.

It is well known that tissues transplanted from one species to another fail to grow except under certain conditions. Such heterologous transplants have been successful in the anterior chamber of the eye and on the chorio-allantois or in the yolk sac of the chick embryo. In specific instances other methods have had limited success.

The fact that many types of tissue have been grown for short periods of time on the chick chorio-allantois may be related to the absence of antibody formation. The absence of complement from the serum of the chick embryo was noted as early as 1907 (1). In 1929, Grasset (2) observed that the chick embryo lacked the ability to produce antibody. Polk, Buddingh, and Goodpasture (3) in 1938 showed that complement for sensitized sheep cells was not present in the serum, extraembryonic fluids, or tissues of the chick embryo before hatching. Just at hatching or immediately afterward it was suddenly present and gradually increased to a maximum in the adult fowl. Murphy (4) in 1914 observed that there was no reaction upon the part of the chick embryo to foreign tissue growing on the chorio-allantois until the eighteenth day of incubation.

The failure of growth of heterologous tissue, either normal or neoplastic, has been thought to be based on immunity reaction and species specificity. Therefore, it seemed reasonable that successful

transfer of heterologous tumor tissue might be effected in the chick embryo if only for a limited period of time. The presence of growth-promoting substances in the rapidly growing chick embryo should make it an ideal medium for growth of heterologous tissue. Furthermore, it seemed that the environment of the tumor tissue within the embryo itself would approximate more closely the environment of such tissue as seen in patients than would tumor growth upon the chorio-allantois or within the yolk sac.

Previous experience of Lee, Stavitsky, and Lee (5) and Lee and Stavitsky (6) with intravenous inoculation of chick embryos with suspensions of *Mycobacterium tuberculosis* suggested that this method might be useful in establishing heterologous tumor growth within the embryo. The use of this technic also offered an opportunity to determine what tissues were most likely to be the site of metastasis following blood stream dissemination.

Sterile tumor tissue was obtained from patients at operation, from tumor-bearing rats and mice, and from tissue culture. The tumor tissue was collected under aseptic precautions. It was then forced through a 70-mesh Monel wire screen, and a sterile suspension of tumor cells in physiological saline was prepared. Lewis (7) has determined that such suspensions contain viable cells.

Chick embryos incubated for 11 days were selected and the eggs candled. A portion of the egg shell over the air sac was removed and the shell membrane carefully stripped off, exposing the chorio-allantois. An inoculum of 0.05 cc. of the tumor-cell suspension was then injected intravenously. The eggs were sealed with Scotch tape, which formed a window through which one could observe the developing chick embryo. The surviving embryos were sacrificed on the twentieth day of incubation, or 9 days after inoculation.

In all, 278 embryos were injected intravenously with 0.05 cc. of sterile tumor-cell suspension in a series of 17 experiments. Following the intravenous injection of physiological saline alone in 11-day-old

* Read at the annual meeting of the American Association for Cancer Research, Atlantic City, N.J., March, 1948.

† This work was carried out in the laboratories of the Henry Phipps Institute, of the University of Pennsylvania.

‡ The equipment was supplied by the Heyden Chemical Corporation.

embryos, 70 per cent survival is expected. Survival following the intravenous inoculation of chick embryos with sterile tumor-cell suspensions averaged about 50 per cent. This survival has been improved recently by the addition of small amounts of penicillin to the tumor-cell suspension, which is allowed to stand at room temperature for $\frac{1}{2}$ hour prior to inoculation into the embryos.

By this technic the C₅₇ strain mouse sarcoma (Table 1) was successfully grown. In 81.7 per cent of surviving embryos injected with this tumor-cell suspension no growth was demonstrated, while 18.3 per cent of surviving embryos showed evidence of tumor growth. In several cases the growth was small, but definite nests of tumor cells could be found histologically in sections of the liver or brain or both (Figs. 1-3). No evidence of tumor metastasis was found in the kidney or spleen. In one instance, although the chick itself appeared to

TABLE 1
THE GROWTH OF RODENT TUMORS
IN THE CHICK EMBRYO

Tumor	Time interval between removal of tumor from source and injection into chick	No. of embryos surviving after intravenous inoculation	No. of takes
C ₅₇ strain mouse sarcoma	1 hour or less	60	11 (18.3%)
C ₃ H strain mouse carcinoma	1 hour or less	18	0
Total		78	11 (14.1%)

be normal in size and development, diffuse nodulation of the liver was observed grossly. On histologic examination, about one-fourth of the liver tissue appeared to be replaced by tumor tissue, and the brain was diffusely invaded by nests of tumor cells. These appeared to be similar to the parent tumor cells. Mitoses were present as was frequent blood vessel invasion. Occasionally a bile canalculus appeared to be invaded by tumor cells.

The C₃H strain mouse mammary carcinoma failed to grow.

Tumor transfer from human beings (Table 2) was accomplished in four instances. Nine and six-tenths per cent of the surviving embryos injected with such tumor suspensions showed microscopic takes in sections of the liver but not in other embryonic tissues. These takes were all small. The tumors from patients which showed evidence of growth within the embryo were two neuroblastomas, a cerebral hemangioblastoma, and a cerebral metastasis secondary to a bronchogenic carcinoma. The lapse of time between removal of the tumor

from the patient and inoculation of the tumor-cell suspension into the chick embryo may be responsible for the few takes and small growth of human tumor tissue within the embryo.

Following the intravenous injection of normal cells from the embryonic chick liver or brain, perivasculär cellular infiltration and occasional focal necroses were seen histologically in the livers of the surviving embryos, but there were no changes suggestive of tumor formation.

TABLE 2
THE GROWTH OF HUMAN TUMORS
IN THE CHICK EMBRYO

Tumor	Time interval between removal of tumor from source and injection into chick (hours)	No. of embryos surviving after inoculation	No. of takes
Neuroblastoma	6	9	1
Chondrosarcoma (cadaver)	4	2	0
Metastatic adenocarcinoma*	30	0	0
Medulloblastoma	7	4	0
Neuroblastoma	7	4	1
Neurofibroma (cerebral)	7	5	0
Hemangioblastoma (cerebral)	2	3	1
Metastatic carcinoma† (cerebral)	3	15	1
Metastatic adenocarcinoma‡	10	1	0
Total	..	43	4 (9.6%)

* Probably from colon.

† Probably from lung.

‡ Probably from cecum.

TABLE 3
SUMMARY

No. of chicks injected I.V. with tumor-cell suspensions	No. of survivors	No. of takes
278	121	15 (12.4% of survivors)

In the entire series of experiments there was evidence of tumor growth in the brain or liver of 12.4 per cent of surviving embryos injected intravenously with tumor-cell suspensions. The neoplastic cells maintained their histologic character in the embryo and closely resembled the parent tumors. This morphologic evidence suggests the probable identity of the transplants with the parent tumors. Experiments are now under way to effect serial transfer of heterologous tumor transplants. In the case of transplants of animal tumors we are attempting to return such transplants to a member of the original host species. Attempts are

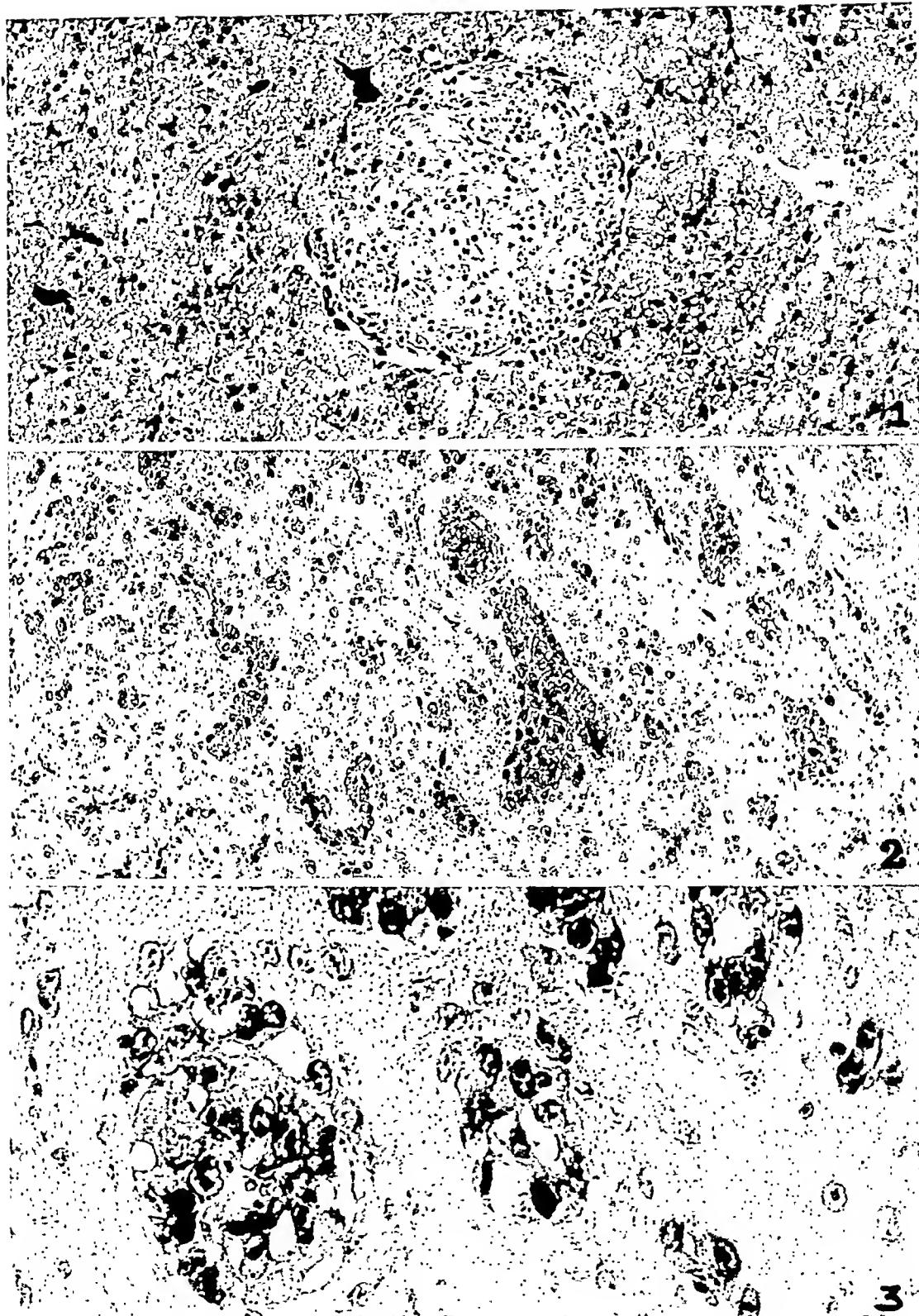


FIG. 1.—Metastatic nodule in the liver of a 20-day-old chick embryo following intravenous inoculation with the C57 strain mouse sarcoma. Mag. $\times 330$.

FIG. 2.—Diffuse involvement of the brain of a 20-day-old

chick embryo injected intravenously with the C57 strain mouse sarcoma. Mag. $\times 160$.

FIG. 3.—Higher magnification of a section of the brain of a 20-day-old chick embryo injected intravenously with the C57 strain mouse sarcoma. Mag. $\times 660$.

also being made to determine what happens to the tumor transplants after the chick is allowed to hatch.

At this time it can be stated that it is possible to grow heterologous tumors by the intravenous inoculation of the chick embryo with sterile tumor-cell suspensions, although we have not been able as yet to propagate tumors by this method consistently.

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CANCER RESEARCH

VOLUME 9

FEBRUARY 1949

NUMBER 2

Some Effects of Long-continued Estrogen Treatment on Male Dutch Rabbits*

HOWARD A. BERN, PH.D.†

(From the Department of Zoology, University of California, Los Angeles 24, California)

That estrogen can play a role in tumorigenesis is a well-established fact (20-22). The observed role of continuous dosage with estrogen for long periods in the induction of genital and extra-genital fibroids in the guinea pig (31), along with the production of fibromyoepitheliomas of the utricular bed of the prostate (33), have led us to conduct similar experiments in male Dutch rabbits. The production of abdominal fibroids is evidently a specific reaction of the guinea pig; such tumors are not obtained in rats, mice, or monkeys (32).

The literature is replete with studies on the effects of estrogen on male sex accessories. Much of the earlier work has been summarized briefly by Moore (35) and Allen, Hisaw, and Gardner (1) and extensively by Gardner (20), Zuckerman (39), Burrows (8), Emmens and Parkes (16), and Bern (2). The extremes of fibromyotropic and epitheliotropic effects on the male sex accessories due to estrogen have been described in the mouse (4, 14, 28), in the rat (26), in the dog (15, 24), in the guinea pig (10, 33), in the monkey (36, 40), in the ground squirrel (38), and in the opossum (9). However, only Lipschütz *et al.* (33), using the guinea pig, reported extensive prostatic fibromyoepitheliomas as a result of uninterrupted exposure to subcutaneously implanted estrogen pellets (for 7 to 11 months).

The rabbit has only occasionally and incompletely been considered in work on the effects of estrogen in the male. In Zuckerman's very extensive 1940 review of the histogenesis of tissues sensi-

* Aided by a Research Grant from the University of California Board of Research.

† Present address: Department of Zoology, University of California, Berkeley 4, California.

tive to estrogens (39), no reference is made to the accessories of the male rabbit. Laeassagne (29, 30) first noted the "pseudohermaphroditic" appearance of the intact male rabbit after estrogenization and described atrophy of the testis and hypertrophy of the epididymis and vas deferens. Frazier and Hu (18) indicated some of the extreme effects of estrogen in intact rabbits, without including histologic description. Their photographs clearly show a decided hypertrophy of the caudal epididymis and the seminal vesicle. The reactions of the rabbit seminal vesicle to estrogen have been studied in some detail by Deanesly (13), who incorrectly considered the gland to be a uterus masculinus, and by Jost (25).

Since the beginning of the study reported herein, the paper of Chevrel-Bodin and Leroy (12) has become available, and our data as reported below are in part confirmatory of the French workers' results. We were particularly concerned with any evidence of possible tumorous growths in rabbits subject to continuous estrogenization for almost 2 years.

MATERIALS AND METHODS

Twelve male Dutch rabbits were used in this study, ranging in age from 11 to 42 months at time of sacrifice. They were kept on a diet consisting of standard commercially prepared pellets, supplemented by greens and/or carrots once or twice a week, and cod-liver oil poured over the food pellets once a week during the latter half of the experiment.

Three of these rabbits (21, 24, and 30 months of age, averaging 2.9 kg. in weight at time of sacrifice) served as intact controls. These rabbits showed accessories no different from those of numerous

younger Dutch rabbits employed as controls in other studies (2) with one exception: the prostates of the older animals were characterized by very large numbers of corpora amyloacea, some of them huge and irregular in shape.

Five of the experimental rabbits were bilaterally castrated at the beginning of the experiment; one was unilaterally castrated, unilaterally cryptorchid; and three remained intact. Castration was performed through the scrotum, and the entire epididymis was removed with the testis. Weighed pellets of estradiol dipropionate were implanted subcutaneously on the inner surface of the right thigh by means of an incision or by a trochar, and

genization was being maintained. It is possible that palpability is not a good criterion for maintenance of hormone supply, inasmuch as Geist, Walter, and Salmon (23) have shown that a thick avascular capsule is formed around subcutaneous pellets of α -estradiol and α -estradiol benzoate in man, which reduces absorption to practically nil after 3 months. However, in our rabbits, external signs (nipples, penis, dewlap) indicated that estrogenization was being continued as long as the pellets were present.

Any tissues suspected of pathologic changes, along with the entire urogenital tract, were fixed in Bouin's fluid and routinely dehydrated. Sample

TABLE 1
RESPONSE OF MALE RABBITS TO LONG-DURATION ESTROGENIZATION

ANIMAL NO.	AGE AT SACRIFICE (MONTHS)	CONDITION OF TESTES	DURATION OF TREATMENT (DAYS)	SEMINAL VESICLE			REMARKS
				MG. ES-TRADIOL DIPROPIONATE ABSORBED	Extent and thickness of metaplasia (μ)	Fibromuscular thickness (μ)	
NC-31	21	Intact control	0	730	4.2
NC-32	24	Intact control	0	630	5.9
NC-30	30	Intract control	0	750	5.1
EST-6	12	Castrate	159	4.6	Complete: 60	1230	20.1
EST-3	11	Intact	191	8.7	Patches: 40	1250	12.9
EST-8	21	Castrate	435	19.3	Complete: 60	2000	14.8
EST-7	22	Intact	444	16.6	Complete: 45	2040	12.0
EST-9	22	Unilateral cryptorchid	466	18.7	Patches: *	1660	18.7
EST-4	24	Intact	582	29.1	Complete: 130	1370	15.2
EST-2	42	Castrate	609	22.1	Complete: 35	1920	11.0
EST-5	27	Castrate	632	29.3	Patches: *	845†	33.0
EST-1	25	Castrate	633	34.9	Complete: 110	1740	18.1

* Too irregular to measure.

† Seminal vesicle enormously distended with secretion.

new pellets were added from time to time. Toward the last half of the experiment α -estradiol pellets were used in place of the ester, but the recorded total hormone absorption shown in Table 1 is computed by converting the weight of the α -estradiol absorbed to the equivalent dipropionate ester weight.

The animals were 4 to 7 months of age at the time that pellets were first implanted, with one exception, EST-2, which was 22 months old. In castrates, pellets were implanted at the time that the testes were removed. All experimental rabbits, except EST-3 and EST-6, which died after 191 and 159 days of treatment, respectively, and EST-4, which had a paralyzed right leg, were in good condition at time of death.

New pellets were implanted when the old pellets were still slightly palpable. It was felt that as long as the pellets were distinctly palpable, estro-

sections of the several organs were cut at 6μ and stained with hematoxylin and eosin. The basis for the nomenclature of the several accessories employed herein has been discussed previously (3).

RESULTS

External appearance.—External evidences of feminization were prominent in all estrogenized animals. These included (1) enlargement of the mammae and nipples, including the production of a watery milk; (2) transformation of the penis into a vagina-like (hypospadiac?) canal and reduction or disappearance of the scrotal sacs; (3) appearance of a prominent, characteristically female, ruff of skin on the neck (dewlap, see Frazier and Mu [19]). In addition, small papillomata on the prepuce, mentioned by Lacassagne (29) as occurring in rabbits after very large doses of estrone, were frequently observed.

Extra-genital viscera.—Although organs other than those of the reproductive system were not studied histologically in detail, certain gross observations were made and confirmed in histologic study. Kidney hypertrophy was evident, and in four animals distinct areas of fibrosis were noted in the kidneys. One of these animals showed a large ecalculus in the right ureter, with calculous deposits in the kidney and tremendous hydronephrosis. Indications of pathologic alterations were found also in some adrenals and livers.

The bladder walls were thickened, and there was an increase in urinary sediment residual in the bladder. Definite fibrosis of the connective tissue mesentery in the rectum-seminal vesicle area occurred, and in two animals hypertrophic changes in the rectum were apparent. Such changes in the rectum have not been reported previously as a sequel to heavy estrogenization. In EST-1, hypertrophy of the individual smooth muscle fibers in the rectum, which appeared "swollen," produced a thickening of the fibromuscular wall to about twice normal. In EST-4, the smooth muscle cells of all the muscle layers (including the muscularis mucosae) were enormously enlarged, resulting in a fibromuscular wall about three times thicker than normal (cf. Figs. 7 and 8). In both cases, the rectal lumen was considerably larger than normal.

No evidence of abdominal fibroid formation was found.

In six of the nine estrogenized rabbits in our series, extensive fat deposits were noted along the vasa deferentia (Figs. 1 and 2) and in the epididymides of the intact animals, as well as in the bladder neck region. Histologic section revealed that these deposits were often intermuscular and subfibrosal and were responsible for some of the generalized hypertrophy of the vasa and the epididymides. Chevrel-Bodin and Cormier (11) reported irregularities in fat metabolism in 7 per cent of a series of estrogenized rabbits. In addition to tumors (*épidome*) at the site of injection of hormone, they observed fatty deposits in the inguinal fold and in the viscera.

Testis and epididymis.—The testes in the three estrogenized intact rabbits and the one unilaterally cryptorchid rabbit were all atrophic and intra-abdominal. Microscopic sections showed two different pictures: the testes of EST-3 and EST-7 consisted of atrophied tubules with little evidence of interstitial tissue; the testes of EST-4 and EST-9 were almost exclusively masses of interstitial cells with a few atrophic tubules.

The caudal epididymis was hypertrophied to huge proportions in the estrogenized intact rabbits (Fig. 1). Some of the tubules were extremely di-

lated and cystic; others were heavily metaplastic. The estrogen-induced metaplasia was generally to a stratified columnar epithelium. The normal caudal epididymis contains efferent ductules, ductus epididymidis, and the coiled portion of the vas deferens, as well as possible Müllerian vestiges; however, we were unable to classify anatomically the tubules in the estrogenized epididymis.

Subfibrosal and intertubular fat deposits were present in the hypertrophic epididymis, which also evidenced a pronounced increase in muscle and connective tissue. In one rabbit (EST-4) a widespread fibroma was found in the epididymis (Fig. 9); in another (EST-9) epithelial proliferations had invaded the connective tissue and the muscle layers. Three rabbits showed some true keratinization of one or more tubules, and lymphocytic and polymorphonuclear infiltration (including intraepithelial abscesses) was evident in all.

Vas deferens.—In all estrogenized rabbits the vasa deferentia were hypertrophic, generally showing discrete swellings of muscle and fibrous tissue along their course, as well as intermuscular and subfibrosal fat deposits (Figs. 1 and 2). Sections revealed either multiple canals or a single large canal lined by a slightly metaplastic epithelium (transitionoid). Polymorphonuclear and lymphocytic infiltration was generally heavy.

In two animals, EST-1 and EST-8, glandular proliferations from the vas deferens had invaded the connective tissue stroma and the muscularis, and small groups of detached epithelial cells were found scattered in the surrounding tissues (Fig. 4). The cancerous nature of these changes is open to question, since their independence of continued estrogenization was not determined. Similar growths in the uterus of the mouse (34) and of the rabbit and guinea pig (37) treated with estrogen (Selye's "experimental invasive endometriosis") have been described. Sections of the vas deferens near the epididymis of EST-9 showed proliferative (and keratinizing) changes probably continuous with those found in the epididymis proper. In addition, the vas deferens of EST-8 showed a prefibromatous nodule, resembling the epididymidal tumor in EST-4.

The ampullary region of the vasa deferentia retained a fairly normal appearance. Although less extensively glandular than in sexually mature rabbits, the ampullae showed infrequent metaplasia, and the main lumen was surrounded by numerous glands lined by a tall columnar, evidently secretory, epithelium. Lymphocytic infiltration was characteristically heavy in the inter- and circum-glandular stroma.

Seminal vesicle.—The most striking effects on

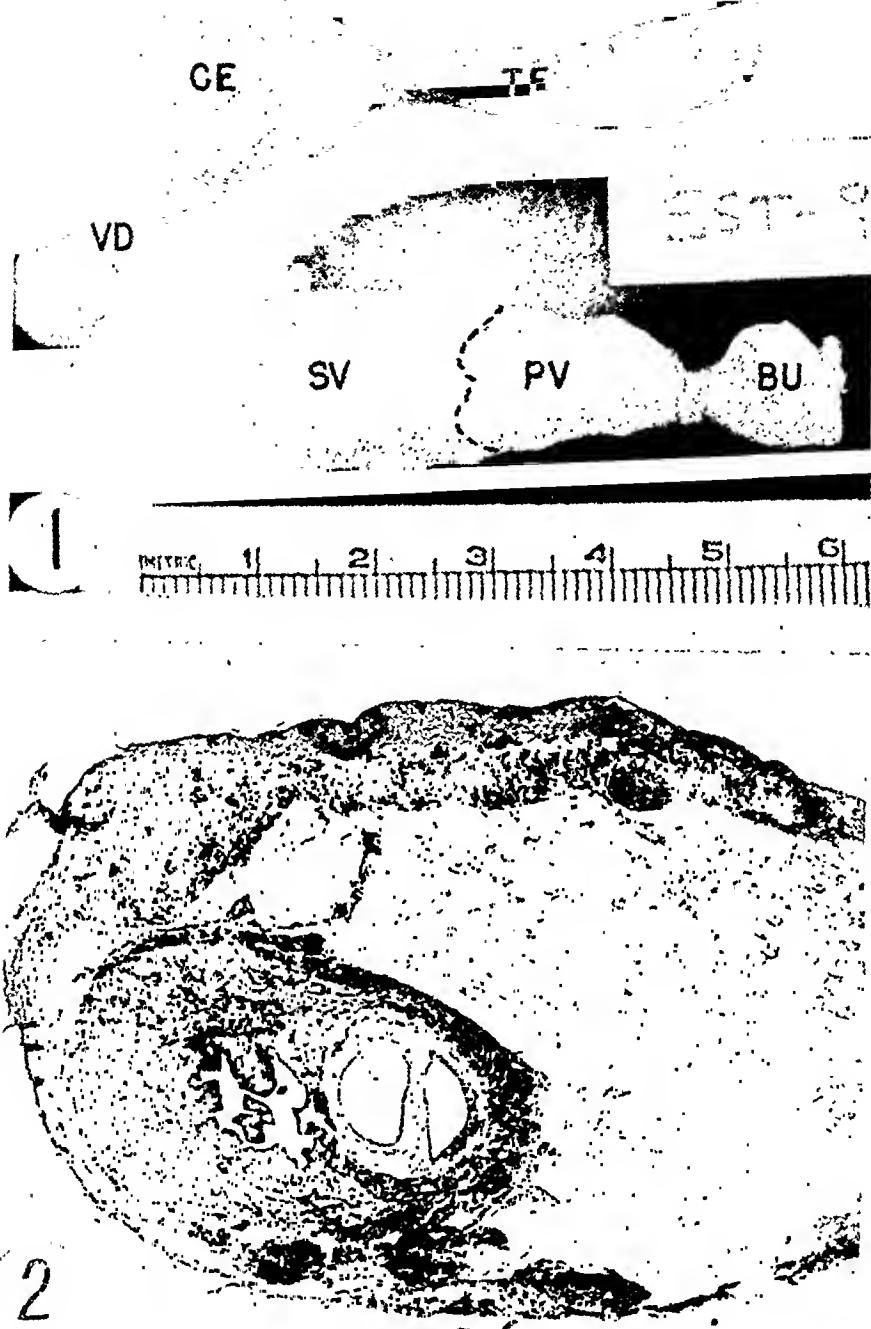
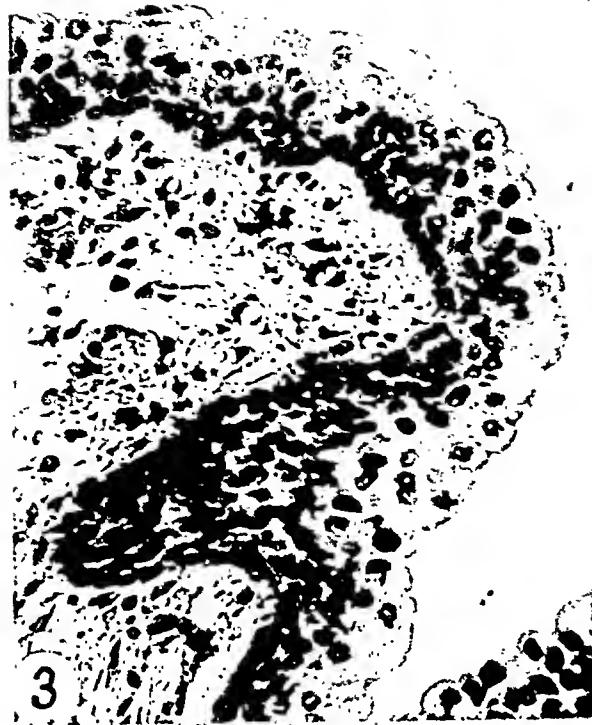


FIG. 1.—Sex accessories and testis-epididymis of 22-month-old unilaterally cryptorchid Dutch rabbit after absorption of 18.7 mg. estradiol dipropionate over a 466-day period. Note seminal vesicle and epididymis hypertrophy and fat-inlosed vas deferens. Dotted line demarcates anterior limit of vesicular gland. Mag. $\times 0.8$. *st.*, seminal vesicle; *pr.*, prostate and vesicular gland; *bu.*, bulbourethral gland;

te., testis; *ce.*, caudal epididymis; *vd.*, vas deferens.

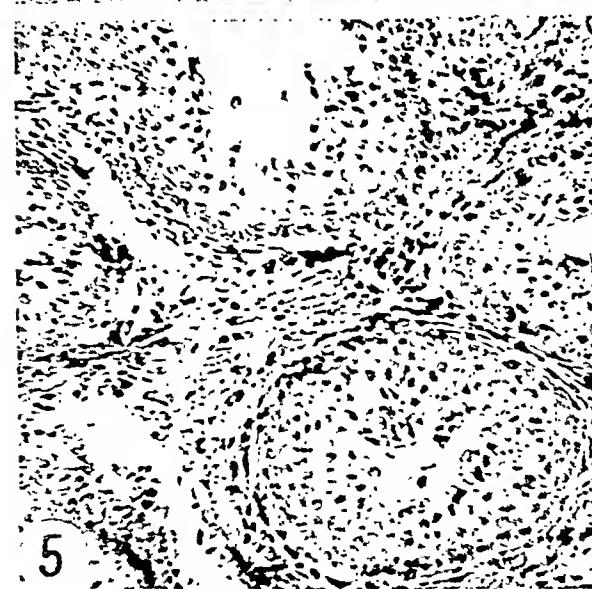
FIG. 2.—Cross-section of vas deferens of 22-month-old intact Dutch rabbit after absorption of 16.6 mg. estradiol dipropionate over a 444-day period. Note intermuscular fat deposits, muscle nodules, and multiple canals (those on the right possibly Müllerian in origin). Mag. $\times 12$.



3



4



5



6

FIG. 3.—Metaplastic epithelium of seminal vesicle of 12-month-old Dutch rabbit, castrate with absorption of 4.6 mg. estradiol dipropionate over a 159-day period. Note "transitionoid," nonkeratinized character. Mag. X400.

FIG. 4.—Area of muscular wall of vas deferens of 25-month-old Dutch rabbit, castrate with absorption of 34.9 mg. estradiol dipropionate over a 633-day period. Note glandular proliferations invading connective tissue and muscle. Mag. X180.

FIG. 5.—Alveoli (type 3) in vesicular gland of 25-month-

old Dutch rabbit, castrate with absorption of 34.9 mg. estradiol dipropionate over a 633-day period. Note adenomatous character; desquamating, but nonkeratinized surface layers. Mag. X180.

FIG. 6.—Alveoli (type 2) in vesicular gland of 27-month-old Dutch rabbit, castrate with absorption of 19.3 mg. estradiol dipropionate over a 495-day period. Note irregularly metaplastic, stratified columnar epithelium. Mag. X180.

the seminal vesicle were the gross increase in size and the fibromuscular hypertrophy (Fig. 1), both extremes of the trends observed in rabbits treated for short periods with estrogens (2). All the estrogenized rabbits of this series possessed a huge seminal vesicle, with apparent stimulation of both muscle and connective tissue. The subepithelial stroma was edematous, but the bulk of the hypertrophy was due to the hyperplastic smooth muscle. Hyperemia was also evident. Table 1 lists some of the data illustrating the definite fibromyotropic and epitheliotrophic effects of estrogen on the seminal vesicle.

Some epithelial metaplasia (Fig. 3) was evident in all seminal vesicles, varying from metaplastic

patches in a columnar epithelium lining as in EST-3 (191 days of treatment) to a completely metaplastic epithelium, 130 μ thick, as in EST-4 (582 days of treatment). This metaplastic epithelium is difficult to classify. It is definitely not stratified squamous and can probably best be described as stratified columnar or "transitionoid."

It should be pointed out that glands of some kind were present in all the seminal vesicles. All except two rabbits showed intraepithelial glands, and all except one possessed modified tubuloalveolar glands. The seminal vesicles possessed one or more of the following types of glands: shallow pits, branched tubules, large active glands lined by tall columnar epithelium, metaplastic alveolar glands,

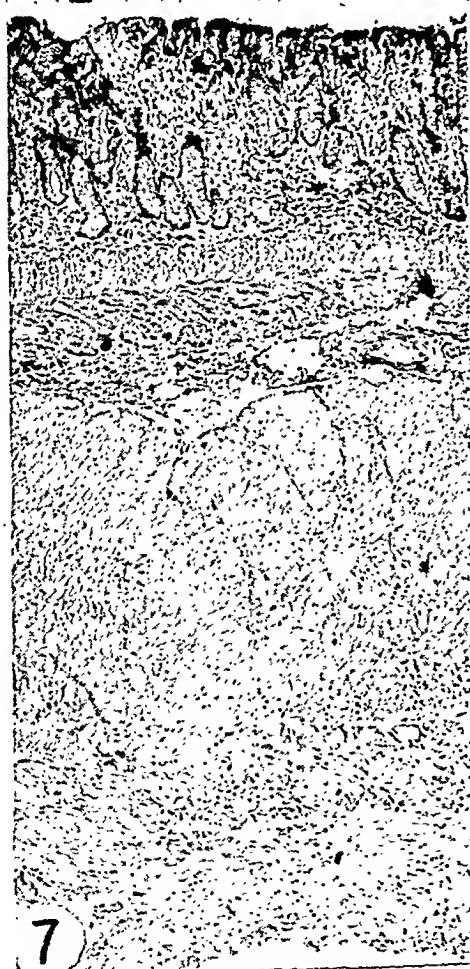


FIG. 7.—Rectum wall of 24-month-old intact Dutch rabbit after absorption of 29.1 mg. estradiol dipropionate over a 582-day period. Note hypertrophy of all muscle layers, including muscularis mucosae (cf. Fig. 8). Mag. $\times 60$.



FIG. 8.—Rectum wall of 21-month-old normal control Dutch rabbit. Mag. $\times 60$.

FIG. 9.—Area of epididymidal fibroma in 24-month-old intact Dutch rabbit after absorption of 29.1 mg. estradiol dipropionate over a 582-day period. Mag. $\times 180$.

huge dilated cysts with flattened epithelium. Secretory material was present in most of these glands.

There was no consistent difference between the main body of the gland and the duct, metaplasia being equally extensive in both. Polymorphonuclear and lymphocytic infiltration was universally present; intraepithelial and glandular abscesses, as well as subepithelial lymphoid aggregations, were common. One animal, EST-9, showed a large sac lined by tall columnar epithelium with densely granular cytoplasm located between the duct of the seminal vesicle and the urethra. This structure was located in the same position as the true uterus masculinus described in a normal albino rabbit (3) and showed evidence of nonmetaplastic epithelial stimulation.

Vesicular gland and prostate.—There was considerable variation in response to estrogens in the vesicular gland and prostate. In general, fibromuscular hypertrophy and hyperemia were present in all. The parenchyma was equal to, or more abundant than, the fibromuscular tissue. In most cases the glands were somewhat smaller than normal, but in two animals the vesicular gland was notably hypertrophic, owing to cystic dilatation of several of the alveoli and to fibrosis and edema of the stroma.

The glandular response was striking in all cases. Four arbitrarily defined alveolar types could be seen in different regions of the gland, generally several types in a single gland: (1) cystic alveoli, lined by a stratified or simple squamous epithelium, with lumina often containing leukocytes and some secretory material; (2) alveoli lined by a disorganized, irregularly metaplastic, stratified columnar epithelium, several layers thick (Fig. 6)—probably transitional between types 3 and 4; (3) heavily metaplastic alveoli (Fig. 5), often solid cords or with extensive papillomatous ingrowths into the lumen, and "adenomatous" in appearance; cells transitionoid or stratified columnar; occasional intraepithelial glands and frequent abscesses; generally terminal to type 4, apparently new growths into the stroma; (4) alveoli lined by simple tall columnar or two-layered epithelium with cells secretory in appearance, with occasional metaplastic patches.

Type 3 alveoli occurred extensively and resembled neoplastic growths in their appearance. Metaplasia in the prostate and vesicular gland was often more profound in the alveoli than in the more proximal ducts. Polymorphonuclears occurred in the lumina and between the epithelial cells in large numbers; lymphocytes were concentrated in the circumalveolar stroma.

No evidence of cornification of the metaplastic epithelium was seen in any of the urogenital structures except the prostatic urethra and certain tubules in the epididymis in some animals. Some of the vesicular gland alveoli in EST-9 showed epithelial sloughing of precornified cells, which probably represents the "cornified" desquamation referred to by Jost (25). Evidently the reaction of the male sex accessories to estrogen in the rabbit does not include true keratinization.

The prostate of EST-4 was nearly normal in appearance, with alveoli lined largely by tall columnar, actively secretory epithelium. This animal possessed a testis showing interstitial cell hypertrophy and may be an instance of acquired resistance to estrogen, as was described in a male mouse by Burrows (7). Several of the estrogenized prostates showed corpora amylacea.

DISCUSSION

The observations reported above are in substantial agreement with those in Chevrel-Bodin and Leroy's experiments (12) on a large series of estrogenized rabbits. We did not observe any great degree of cystic dilatation of the rete testis; however, both the tubular and the interstitial cell types of testes were found. Interstitial cell hypertrophy has also been reported in mice after treatment with certain estrogens (5). In the epididymis, we observed a fibroma and invasive epithelial growths, as well as fibromuscular hypertrophy, cystic dilatation, and metaplastic tubules. Similarly, in the vas deferens, in addition to the fibromuscular overgrowth, we noted one presfibroma and two instances of invasive epithelial growths. Chevrel-Bodin and Leroy pointed out extensive metaplastic and proliferative lesions in the ampullae, which we found were least affected of all the accessories and ducts in our rabbits.

Our descriptions of the epithelial and fibromuscular changes in the seminal vesicle, prostate, and vesicular gland are consistent with the earlier work. Chevrel-Bodin and Leroy concluded that the groups of solid cords observable in the prostate and vesicular gland (not distinguished in their report) represented true adenomatous lobules.

It is obvious that the effect of estrogen on the accessories is universally stimulatory. Both the fibromuscular and the epithelial components reacted to the stimulus. The normal secretory epithelium was generally lost, but some secretory activity was apparent in the replacing epithelium. The distended seminal vesicle of EST-5 (33 mm. in width) was particularly noteworthy in this regard.

The extensive metaplasia occurring in the pros-

tate, vesicular gland, and seminal vesicle is different in the rabbit from that seen in other mammals. Keratinization does not characterize the metaplastic epithelium, and the changed epithelium is not stratified squamous but stratified columnar or transitionoid. Nodular keratinized lesions¹ occur spontaneously in the prostates and vesicular glands of our Dutch rabbits, indicating that these accessories are capable at some time of producing a stratified squamous epithelium and keratin. However, after estrogenization (in either castrate or intact animals) the metaplasia is adenomatous, resembling the seminal vesicle epithelial changes observed in the rat by Korenchevsky (27) after treatment with estradiol dipropionate plus androsterone or dehydroandrosterone.

Cornification was observed in the hypertrophic epididymis accompanying metaplastic changes of the epithelium. While it is possible that this represents a spontaneous pathologic change, such as is seen in the normal prostate and vesicular gland, such changes have not been observed previously in any incidental studies of the epididymides of normal rabbits. In any case, this occurrence of cornification in areas far removed from the urogenital sinus epithelium (as in the keratinization of epididymidal Müllerian duct vestiges in the mouse [6]) does not conform with Zuckerman's (39) concept of the possible participation of urogenital sinus epithelium in all genital squamous metaplasia.

The reasons for the zoological specificity of the reactions to estrogen in different animals are not known. Fibromyoepitheliomas characteristic of the estrogenized guinea-pig prostate (33) were not seen in the rabbit. The squamous metaplasia characteristic of estrogenized accessories in the mouse, rat, opossum, etc. (4, 26, 9), was also not seen in the rabbit. In addition to the possibility of tissue specificity, the adrenal in the rabbit (and the atrophic testis in intact estrogenized animals) may produce a testoid which modifies the tissue response to exogenous estrogen to prevent squamous metaplasia and keratinization. It is also possible that the liver may metabolize the estrogen in a manner different in the rabbit from that in other mammals.

The epididymis-vas deferens system in the rabbit is worthy of special attention because of the several instances of tumorigenesis observed therein (two fibromas, three invading epithelial proliferations). It is possible that the tendency to uterine-like invasive proliferations may be ascrib-

able to the presence of Müllerian duct rests or vestiges in the epididymis and vas of the rabbit. The multiplicity of canals in the estrogenized vas (Fig. 2) might indicate this. Burrows (6) pointed out the ease with which such vestigial structures become cystic and keratinized after estrogenization in male mice. Two cases of adenofibromyoma of the epididymis, with hormones possibly playing an etiologic role, were recently described in man by Falconer (17). The epididymal enlargement described by De Jongh *et al.* (15) in the dog, however, was due to nontumorous fibromuscular hypertrophy.

As pointed out above, no abdominal fibroids were observed; however, the fibromatous growths seen in the vas deferens-epididymis system in two of the animals of this series indicate a possible specific reaction to estrogens on the part of certain genital tissues in the male rabbit. These growths were grossly apparent and were studied in sample sections through the caudal epididymis and vas deferens; serial sections might have revealed other signs of possible neoplasia.

SUMMARY AND CONCLUSIONS

A series of male Dutch rabbits, intact and castrate, was subjected to continuous treatment with estradiol and estradiol dipropionate for periods of 159 to 633 days.

Dramatic change occurred in the sex accessories and in the duct system. The seminal vesicles were tremendously enlarged, and the epithelium of the prostate, vesicular gland, and seminal vesicle underwent metaplasia to a nonsquamous, transitionoid type, adenomatous in appearance.

No abdominal fibroids or prostatic fibromyoepitheliomas, as reported in the guinea pig, were found; but tumorous changes (fibromas and invasive epithelial proliferations) hitherto undescribed in male mammals after estrogenization were noted in the vasa deferentia and in the epididymides of certain rabbits. In addition, striking hypertrophy of the rectum was seen in two rabbits.

ACKNOWLEDGMENTS

The author wishes to express his appreciation to Professor Boris Krichesky for his valuable suggestions in regard to this study. Dr. W. A. Wright, of Schering Corporation, generously supplied the α -estradiol pellets and Ciba, Incorporated, the estradiol dipropionate.

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¹ These spontaneous lesions, morphologically resembling those of avitaminosis-A in other animals, are being investigated further.

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Histological Appearance of Mouse Sarcoma 180 Infected by Vaccinia Virus*

ROBERT A. KRITZLER, M.D., BARBARA MULLIKEN, AND JOSEPH C. TURNER, M.D.

(From the Department of Medicine, College of Physicians and Surgeons, Columbia University,
and the Presbyterian Hospital in the City of New York)

INTRODUCTION

Levaditi and Schoen, in 1936, demonstrated Guanieri bodies in the tumor cells of the Shope papilloma infected by vaccinia virus and noted infiltration of the tumor by polymorphonuclear leukocytes (6). Levaditi and Haber found that the introduction of fowl pest virus into mice epithelioma resulted in massive necrosis of the tumor cells (5). However, the virus was lethal to the host, and similar necrosis occurred in liver cells.

No characteristic changes were found by Findlay and MacCallum in mouse carcinomas infected by the yellow fever virus (2). Andrews observed typical Virus III intranuclear inclusions in the cells of a rabbit fibroma (1). Tumors carrying Virus III regressed more rapidly than did the control fibromas. Intranuclear inclusions were found in areas of tumor necrosis but were also widespread in tumor cells which revealed no other alteration or necrosis.

In a previous communication from this laboratory, Turner and Mulliken reported that transplants of mouse sarcoma 180 infected with vaccinia virus harbored the virus in high titre for extraordinarily long periods of time, in contrast to normal tissues of the host, and that the infected tumors grew more slowly and less successfully than they otherwise would have (7). The explanation offered was that the virus acts directly on the neoplastic cells. The report which follows is concerned with the histological study of some of these tumors to determine whether there was anatomical evidence of the presence of the virus in the tumor cell or of an effect of the virus on the tumor cells or on its environment.

EXPERIMENTAL

Grafts of mouse sarcoma 180 infected 6 days previously with vaccinia virus were made subcutaneously in mice. The animals were killed at intervals of 4 to 36 days thereafter. The details of

the experiments are recorded in an earlier communication (7). A histological study was made of 34 infected and of 37 noninfected tumors.

RESULTS

All but 2 of the infected tumors were shown by intradermal tests in rabbits to contain vaccinia. No specific histologic evidence of tumor parasitization by the vaccinia virus was obtained. Guanieri and elementary bodies were not demonstrable in Giemsa preparations, either in the tumor cells or in the supporting tissue or capillary endothelium.

Since growth was impaired in infected tumors, it was of importance to determine whether the effect might be due to an inflammatory reaction within or around the tumor, such as that seen in the skin and subcutaneous tissues of the mouse or rabbit after intradermal injection of virus. No appreciable inflammation attributable to the presence of virus could be found in the tumors. Capillary engorgement, varying edema, and diffuse infiltration of the tumor by polymorphonuclears was observed during the first 10 days after transplantation in most of the tumors, of both the infected and the noninfected groups. Necrosis of inflammatory and capillary endothelial cells as seen in the vaccinal reaction in the mouse tail was not present in the infected tumors. After the end of the second week following transplantation, the tumors of both groups were free of inflammatory reaction, except in the areas of necrosis which occur regularly in the course of development of sarcoma 180. Thus tumors known to harbor the virus in high titre in the second and third weeks showed no inflammatory reaction to the virus.

Mitotic figure counts were done to determine whether the impairment of growth in the infected tumors might be associated with a change in cell division. The total number of mitotic figures in ten random ($\times 1,500$) microscopic fields in each infected tumor was compared with a similar count for each noninfected tumor. During the first 10 days after transplantation the mitotic figures were

* These investigations have been aided by a grant from the Jane Coffin Childs Memorial Fund for Medical Research.

significantly fewer in infected, as compared with the control, tumors (Table 1).¹ After the tenth day there was no difference in the number of mitotic figures in the two groups.

No alteration of tumor-cell structure was present in infected tumor which could not be seen to some degree in the noninfected tumors as well. However, it is of interest that the occurrence of multinucleated tumor cells and of variation in the size and shape of the nuclei, as well as in the condensation of nuclear chromatin, and in the shape of the cells was more common in the infected tumors. This was particularly true during the first 2 weeks after transplant. The difference was less striking later.

Histochemical examination of the tumors for alkaline phosphatase and for acid phosphatase by the methods of Gomori (3, 4) revealed no differ-

controls. This, together with the fact that the virus has no lytic effect on the tumor cells, suggests that the inhibitory effect of parasitization may be on some metabolic process leading up to cell division.

The more frequent occurrence of anaplastic cells in the infected tumors is paradoxical, since lack of differentiation is usually characteristic of rapid growth. At the present time no explanation is offered.

SUMMARY

Transplants in mice of mouse sarcoma 180, parasitized by vaccinia virus and found to grow less well than control transplants, revealed no Guanieri or elementary bodies in the tumor cells or surrounding stroma. In infected tumors there was no inflammatory reaction which might be attributed to the presence of the virus.

TABLE 1
TOTAL NUMBER OF MITOTIC FIGURES IN TEN RANDOM MICROSCOPIC ($\times 1,500$) FIELDS OF INFECTED AND CONTROL SARCOMA 180

	DAY 4 TO 6*			DAY 7 TO 8			DAY 9 TO 10		
	Tumors examined	Range	Mean	Tumors examined	Range	Mean	Tumors examined	Range	Mean
Control	6	4-18	9.5	8	9-17	13.6	6	10-16	13.3
Infected	8	1-6	3.8	8	4-12	8.8	9	7-17	10.5

* After transplant.

ence in the amounts of these enzymes in the nuclear chromatin or mitotic figures of infected and control tumors.

DISCUSSION

The absence of inclusion and elementary bodies from the tumor cells does not exclude the hypothesis that the virus has an affinity for them. The cells of a sarcoma, such as 180, are presumably derived from mesenchyme. They might therefore differ from epidermal cells in their structural reaction to virus, e.g., formation of Guanieri bodies. Against the idea that the virus simply remains only in the supporting stroma of the tumor is the fact that it disappears rapidly from normal subcutaneous tissues of the mouse, whereas it persists in the tumors for as long as 31 days after transplant. Moreover, no inclusion or elementary bodies were found in connective tissue or endothelial cells.

The presence of fewer mitotic figures in infected tumors is in keeping with the observation that such tumors grow less well than the noninfected

The differences observed between infected and noninfected tumors were quantitative rather than qualitative. Mitotic figures in infected tumors were fewer than in control tumors during the first 10 days after transplant. Variations in differentiation occurred in control and infected tumors but were more common in the latter.

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¹ The authors are indebted to Miss Evelyn Elveback, of the Department of Biostatistics, School of Public Health, for analysis of the data. By the chi-square method the difference between the number of mitotic figures in the two groups is found to be significant at the 1 per cent level.

Primary Liver-Cell Carcinoma (Hepatoma) in the Dog*

R. M. MULLIGAN, M.D.

(From the Department of Pathology, University of Colorado Medical Center, School of Medicine, Denver 7, Colorado)

Although nodular hyperplasia of liver cells is fairly common in the dog (3, 6, 8, 12, 14), true neoplasia of liver cells is relatively unusual. In this laboratory, nodular hyperplasia of liver cells (12) was observed in 10 out of 61 autopsied dogs (Fig. 1). Of the 45 cases of epithelial cancer of the liver found in the literature (1-7, 9-11, 13, 15-17), 35 were typed as carcinoma, presumably of liver-cell origin; 8 were classified as adenocarcinoma, evidently of bile duct origin; and 2 were not studied histologically. In 10 cases a definite statement concerning metastases was made. Of the 5 carcinomas, 2 produced metastases and 3 remained within the liver. Of the 5 adenocarcinomas, 4 metastasized and 1 did not. In 11 cases, the sex was female in 7 and male in 4. In 7 dogs the age ranged from 8 to 15 years. Breed was as follows in 10 cases: fox terrier, 2; pinscher, 2; and dachshund, Yorkshire terrier, French bulldog, "sheep hound," Airedale terrier, and German shepherd, 1 each.

REPORT OF FOUR CASES OF HEPATOMA IN THE DOG

Case 1 (47R-378).—A female chow, 10 years old, a patient of Drs. J. R. Naylor and L. C. Smith, showed a neoplasm of 1,335 gm., 18 × 14 × 14 cm., on the inferior surface of the liver, arising mainly from the caudate lobe but also from the posterior part of the quadrate lobe. Cut section revealed a gray-brown fluid and friable red-tan tissue in an eccentric cavity, 10.5 cm. in greatest diameter, within the tumor, which consisted of nodules 2 to 55 mm. in diameter, soft to firm, light-brown, and sometimes red mottled (Fig. 2). The anatomic diagnoses were: carcinoma (hepatoma) of the liver, fibroadenocystic disease of the right fifth mammary gland, follicle cysts of the ovaries, cystic glandular hyperplasia of the endometrium, nodular cortical hyperplasia of the adrenal glands, thrombosis of an esophageal vein, and extra-medullary myelopoiesis of the spleen.

Case 2 (47R-629).—A spayed female "toy shepherd," 12 years old, a patient of Drs. L. R.

Phillips and B. S. Burkhardt, had a 585-gm. tumor arising from the inferior and anterior surfaces of the quadrate lobe of the liver, entirely resected at operation; but the dog was destroyed at the request of the owner. The tumor was fairly firm, bulged on section, and consisted of large lobules of light-brown to tan, sometimes dark-red or tan and dark-red nodules, 4 to 40 mm. in diameter, separated by tough interlacing gray tissue bands (Fig. 3). The anatomic diagnoses were: carcinoma (hepatoma) of the liver, cortical adenoma of the left adrenal gland, nodular cortical hyperplasia of the right adrenal gland, mucous cysts of the gall bladder, and hemosiderosis of the spleen.

Case 3 (48R-107).—A male German shepherd, 14 years old, a patient of Drs. H. G. Weigand and W. J. Shay, had a hepatoma, which, with the liver, weighed 1,720 gm. (Fig. 4), the liver being one-tenth the total. The tumor was 173 × 132 × 125 mm. and arose from the inferior surface of nearly the entire liver, especially from the quadrate and caudate lobes. The cut section was fairly firm, coarsely lobulated, red-tan, and focally red mottled. The individual lobules were finely nodular. The anatomic diagnoses were: carcinoma (hepatoma) of the liver and nodular lymphoid hyperplasia of the spleen.

Case 4 (48R-196).—A male fox terrier, 15 years old, a patient of Dr. F. T. Candlin, showed a liver weighing 708 gm., coarsely lobulated and deeply fissured by dense gray tissue bands (Fig. 5). On cut section the lobules consisted of nodules 3 to 32 mm. in diameter; firm to spongy; tan, green, brown, pink-gray, or white. The anatomic diagnoses were: carcinoma (hepatoma) of the liver, cirrhosis of the liver, carcinoma of the perianal glands, nodular cortical hyperplasia of the adrenal glands, and jaundice.

MICROSCOPIC OBSERVATIONS

Cirrhosis.—The hepatic (central) veins were easily identified. The periportal areas showed both relative and absolute increase of fibrous connective tissue, bile ducts, venules, and arterioles. Complete obliteration of any semblance of portal triads

* This investigation was aided by a grant from the National Cancer Institute of the United States Public Health Service.



FIG. 1



FIG. 2



FIG. 3

FIG. 1 (48R-609).—Nodular hyperplasia of liver, inferior surface. Left edge at top, anterior edge at left. Large sharp defect in left posterior lobe in area cut for tissue sections. $\times 9/20$.

FIG. 2 (47R-378).—Case 1. Hepatoma. Cut section with

eccentric, cystic cavity at top. Surface with stretched, opened common bile duct over right edge at bottom. $\times 9/20$.

FIG. 3 (47R-829).—Case 2. Hepatoma. Cut section of two levels to show large lobules divided into smaller nodules. $\times \frac{1}{2}$.

FIG. 4 (48R-107).—Case 3. Hepatoma. Anterior view. Liver at top. $\times 2\frac{1}{2}$.



FIG. 4



FIG. 5

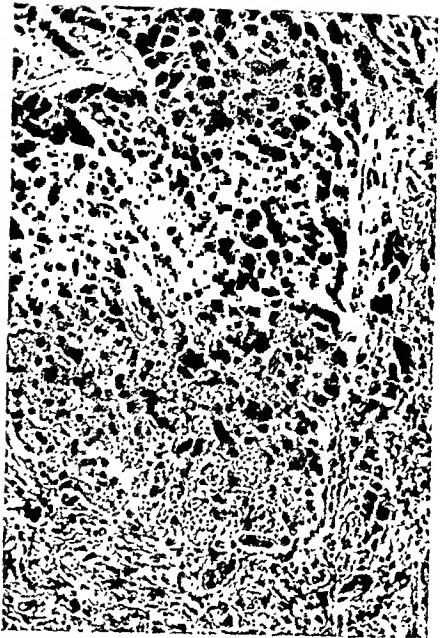


FIG. 6

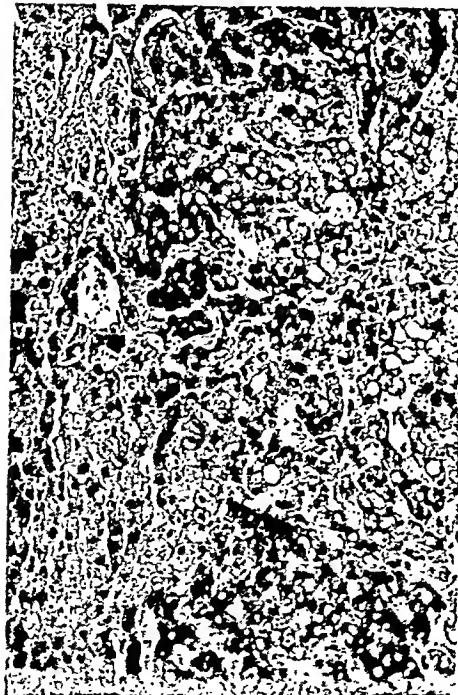


FIG. 7

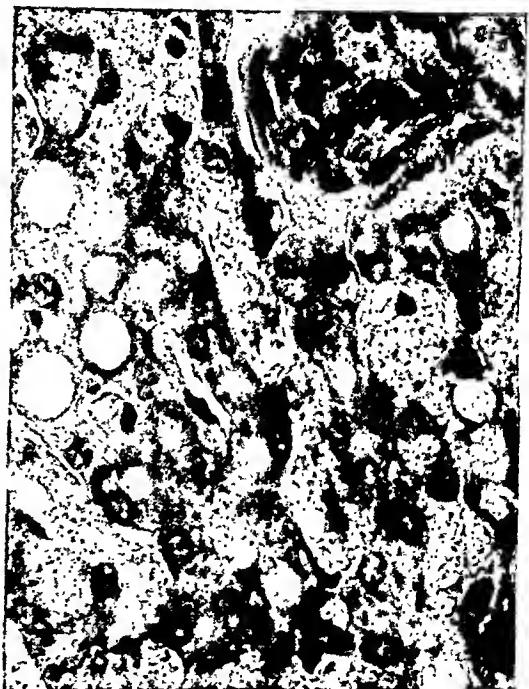


FIG. 8

FIG. 5 (48R-196).—Case 4. Hepatoma and cirrhosis. Cut section of one level in top two pieces and at another level in bottom piece. $\times 1/2$.

FIG. 6 (48R-196).—Case 4. Cirrhosis of liver. Central vein of distorted lobule in upper left-hand corner. Proliferated bile ducts in fibrous connective tissue to right and at bottom. $\times 150$.

FIG. 7 (47R-565).—Nodular hyperplasia of liver. Edge of single nodule in right three-quarters, with retained portal triad in upper right-hand corner and fat vacuoles in several hyperplastic liver cells. Compressed liver cords and sinusoids in left one-quarter. $\times 150$.

FIG. 8.—Higher power of Fig. 7, with portal triad in upper right corner. $\times 600$.

was produced by the merging of adjacent periportal areas (Fig. 6). The distorted single lobules were reduced in size to about half that of the lobules of a normal liver. The polyhedral or rounded liver cells were arranged in single or double rows, but irregularly as compared to those in a normal liver. The cell borders were separated from each other and usually clearly defined or were less commonly hazy and closely approximated. The cytoplasm was finely granular and acidophilic. The often eccentric nuclei were usually single, but 3 or 4 binucleated forms could be seen in a $\times 440$ field. The sinusoids were warped and uneven in conformity to the disarrangement of the cords of liver cells; the Kupffer cells and bile canaliculi were stuffed with bile pigment. The nuclei of the liver cells were round, and nuclear membrane was heavy. The chromatin was finely beaded but unevenly dis-

plasm of the cells was granular, acidophilic, or polychromatophilic and sometimes coarsely vacuolated by fat or finely foamy with glycogen. The nuclei were often single, but a $\times 440$ field contained 4 or 5 binucleated cells. The nuclei were round, and the nuclear membrane was heavy. The chromatin was finely beaded and irregular. Nucleoli were conspicuous, acidophilic, usually single, sometimes double, and often encircled by condensed chromatin. The nuclei within the nodules were 7.5 to 9.1 μ in diameter; those in the surrounding liver cells were 6.7 to 7.4 μ in diameter.

Hepatoma.—The lobules of neoplastic cells directly compressed the adjacent liver tissue (Fig. 9) with flattening of liver-cell cords, sinusoids, and portal triads and with distortion of lobules; or the lobules of neoplastic cells were separated by bands of vascularized connective tissue from each other

TABLE 1
TEN CASES OF NODULAR HYPERPLASIA OF LIVER CELLS IN THE DOG

Number	Accession number	Age	Sex	Breed	Nodules in liver
1	47R-565	16	M	Mongrel German shepherd	8, tan or red-tan, 5-18 mm.
2	47R-684	14	F	Collie	24, light tan to dark red, 5-25 mm.
3	47R-703	13	M	German shepherd	3, red, 15-62 mm.
4	48R-74	12	M-e	Collie	9, tan to dark red, 8-20 mm.
5	48R-95	12	M	Boston terrier	1, light tan, 20 mm.
6	48R-109	12	F-s	Pekingese	6, tan, averaging 6 mm.
7	48R-116	12	F-s	Mongrel German shepherd	Multiple, 1-24 mm.
8	48R-183	7	F	Fox terrier	1, light tan, 20 mm.
9	48R-223	14	M	Collie	4, fairly firm, 9-22 mm.
10	48R-406	11	M	Bull terrier	1, tan, 10 mm.

tributed. The acidophilic nucleoli were usually discrete, sometimes enlarged and more prominent than normal, and occasionally numbered two to a nucleus. The chromatin net tended to be clumped around the nucleoli. The nuclei measured 6.7 to 7.8 μ in diameter, as compared with normal, 6.2 to 7.1 μ .

Nodular hyperplasia of liver cells.—A summary of 10 cases of nodular hyperplasia of liver cells studied in this laboratory is given in Table 1. Each nodule, whether single or multiple, large or small, consisted of irregular liver lobules with retention of the arrangement of central veins and portal triad in each lobule. The liver cells at the margins of the nodules showed compressive flattening, sinusoidal collapse, and distortive displacement of portal triads (Fig. 7). No increase in fibrous connective tissue was seen in the nodules of hyperplastic liver cells or in the adjacent liver tissue. Within the hyperplastic nodules the polyhedral liver cells had indefinite borders and were arranged in interlocking cords, which varied from a single to many cells thick to large solid masses without intervening sinusoids (Fig. 8). The cyto-

and from the surrounding liver parenchyma. In the latter instance, adjacent liver cells were severely atrophic or absent, and bile ducts, venules, and arterioles were entrapped in heavy fibrous connective tissue. The lobules of hepatoma consisted only of neoplastic cells and proliferated vascular spaces resembling sinusoids (Figs. 10, 11, and 12). Portal triads were entirely lacking within the lobules of the hepatoma. The huge neoplastic cells were arranged in solid masses, their borders were usually discrete, their shape was polyhedral or round, and their cytoplasm was granular or slightly to moderately vacuolated or both. The round or oval nuclei varied greatly in size and were usually single, but as many as 5 or 6 binucleated forms were noted in a $\times 440$ field. The nuclear membrane was heavy, and the chromatin was finely beaded or coarsely stippled and irregular. The acidophilic nucleoli were often single, sometimes numbered 2 or 3 to a nucleus, were frequently large and surrounded by condensed chromatin, and displayed scattered mitotic figures. In case 1, the nuclei of the neoplastic cells ranged from 7.5 to 10.7 μ in diameter, whereas the nuclei of the liver cells next

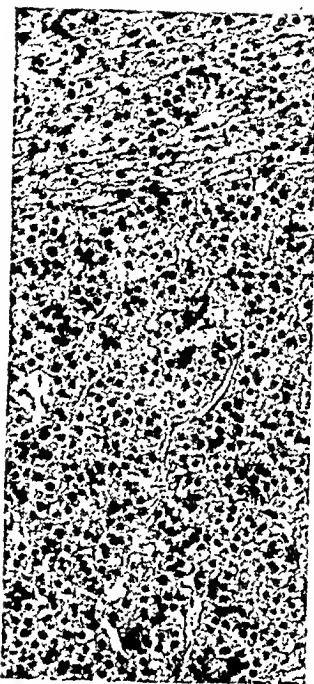


FIG. 9

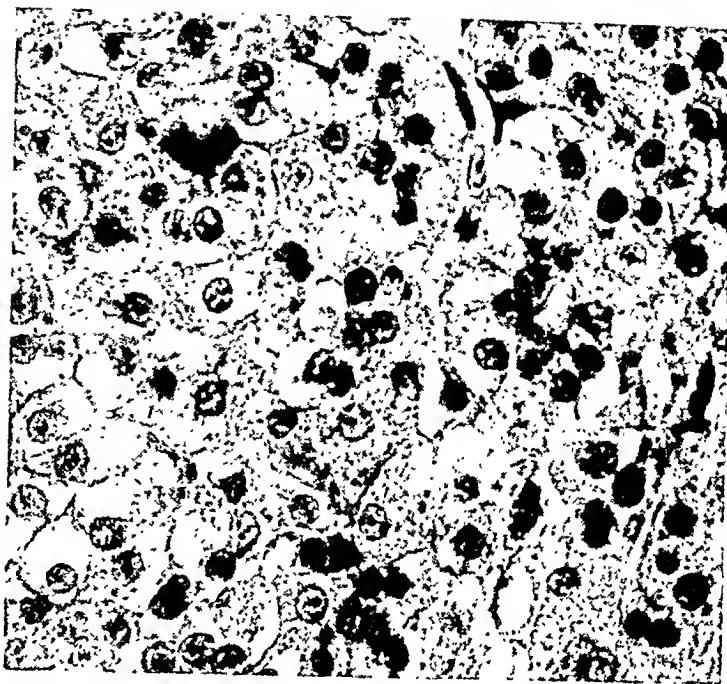


FIG. 10

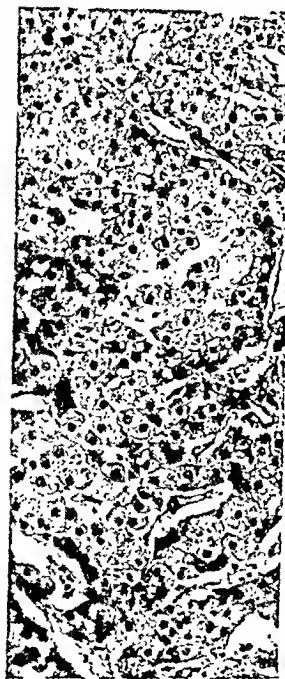


FIG. 11

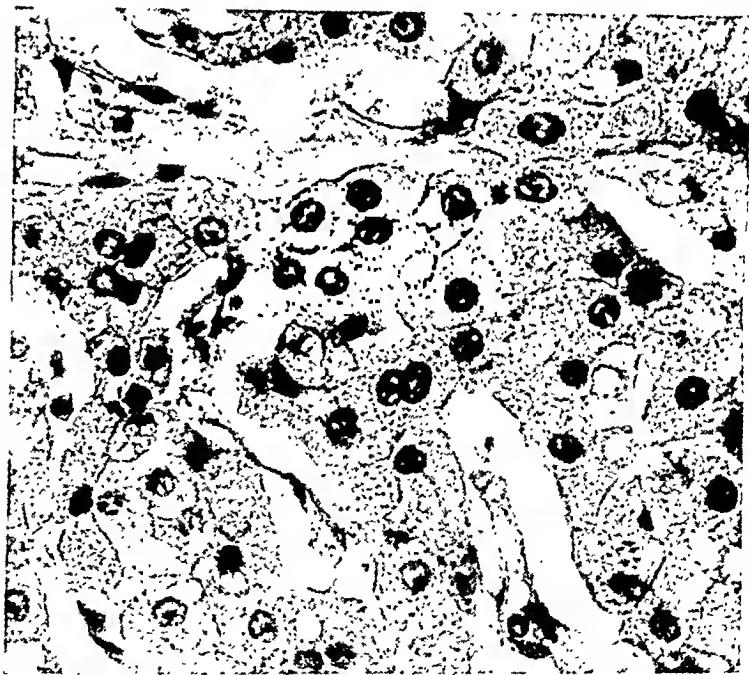


FIG. 12

FIG. 9 (47R-629).—Case 2. Hepatoma in lower three-quarters and compressed liver cords and sinusoids in upper one-quarter. $\times 150$.

FIG. 10.—Higher power of Fig. 9, showing area in middle of hepatoma. $\times 600$.

FIGS. 11 and 12 (47R-378).—Case 1. Hepatoma. $\times 150$ and $\times 600$.

to the neoplasm were 6.5 to 8.5 μ in diameter. In case 2 the nuclei of the neoplastic cells were 6.2 to 8.5 μ in diameter.

SUMMARY

1. A review of the literature revealed 35 cases in the dog of carcinoma, presumably of liver-cell origin; 8 of adenoearcinoma, evidently of intrahepatic bile duct origin; and 2 hepatic cancers not studied histologically. Data on age, sex, and breed were too incomplete to be conclusive.

2. In a group of 4 cases of hepatoma in the dog, inappetence, vomiting, weight loss, emaciation, and an upper abdominal tumor were symptoms noted 1 to 6 months before euthanasia. Resection of one hepatoma was successful and probably would have resulted in complete cure if the dog had been allowed to live. Resection might have been curative in 2 of the other 3 dogs with hepatoma if operation had been made earlier.

3. Three of the hepatomas arose from the inferior surface of the liver near the mid-line, displaced adjacent organs and tissues weighed 585 to 1,550 gm., were separated from the liver by fibrous connective tissue or directly impinged on compressed liver parenchyma, and consisted of large, soft to firm, tan, light-brown, tan-red, or dark-red lobules divided into nodules 2 to 55 mm. in diameter. The fourth hepatoma was associated with advanced cirrhosis of the liver and with atypical proliferation of bile ducts and sinusoids. The dog affected had had severe icterus during life. Microscopically, the lobules of hepatoma consisted of very large neoplastic liver cells in solid masses intimately related to proliferated vascular spaces resembling sinusoids. Portal triads were entirely lacking within the lobules of the hepatoma. The neoplastic cells had granular or vacuolated acidophilic cytoplasm and round or oval nuclei varying greatly in size, fairly frequently numbering two to a cell, displaying occasional mitosis, and supplied with beaded irregular chromatin and large acidophilic nucleoli. No case showed metastasis.

4. Ten cases of nodular hyperplasia of liver cells were compared with the 4 cases of hepatoma. The nodules were usually multiple, fairly firm, tan, red, red-tan, or dark red, and were 5 to 25 mm. in diameter, or varied extremely from 1 to 60 mm. Microscopically, the liver tissue around the single nodules was compressed, but fibrous connective tissue was not increased. The nodules consisted of irregular liver lobules with retention of central

veins and portal triads in each lobule. The polyhedral cells within the nodules were arranged in cords one to many cell layers thick, with distortion of the intervening sinusoids. The cells of the nodules were smaller in both cytoplasmic and nuclear volume and exhibited less histologic evidence of nuclear activity than did the neoplastic cells of the hepatomas.

5. Both hepatoma and nodular hyperplasia of liver cells occurred in dogs in advanced senility or 11 to 16 years of age. Sex preponderance was not striking in either condition, and data on breed were inconclusive.

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The Effects of Casein and Methionine on the Retention of Hepatic Riboflavin and on the Development of Liver Tumors in Rats Fed Certain Azo Dyes*

A. C. GRIFFIN, † Ph.D., C. C. CLAYTON, ‡ M.S., AND C. A. BAUMANN, Ph.D.

(From the Departments of Biochemistry and Cancer Research, University of Wisconsin, Madison, Wisconsin)

That the development of hepatic tumors in rats fed azo dyes can be varied by diet is a well-established fact; but, in spite of extensive studies on individual nutrients, the relation of dietary protein to the process is not particularly clear. The crude materials that have been found to be effective in retarding the development of these tumors are usually high in protein, e.g., liver (18, 21), certain concentrates from liver (8, 18, 27), yeast (18, 21), spleen (3), milk (9), egg white (7, 12, 25), stock diet (18), and others; but these materials also contain other nutrients, such as riboflavin, that might be active in retarding tumor development.

The importance of riboflavin in preventing hepatic tumors due to *p*-dimethylaminoazobenzene (DAB) has been shown in experiments with synthetic diets containing 12 or 18 per cent of casein. Such tumors developed rapidly when the diet contained 2 µgm. of riboflavin per gram but developed very slowly when 20 µgm. of riboflavin were present per gram of diet (20). The ingestion of DAB results in a decrease in the concentration of riboflavin in the liver (10), and the extent of the decrease has been found to be proportional to the carcinogenicity of the azo dye fed (4). Furthermore, when a given azo dye was incorporated into diets known to accelerate or retard tumor formation, the amount of riboflavin retained by the liver tended to be greatest on the protective diets and least on those on which tumor formation is rapid (5). It would therefore appear that the formation of hepatic tumors is in some way associated with subnormal concentrations of riboflavin in the liver and that diets that promote riboflavin retention should increase the resistance of the animal to the

process. Sarett and Perlzweig (23) and others have shown that the riboflavin content of the livers of rats fed equivalent amounts of the vitamin tends to vary with the level of protein intake, and this effect of protein has also been observed in animals ingesting DAB (4). The addition of methionine to diets low in protein has also been reported to increase the storage of hepatic riboflavin (22, 31). This suggests that diets high in protein or methionine might modify the development of liver tumors by promoting a better retention and storage of riboflavin in the liver.

Kensler *et al.* found that riboflavin by itself was ineffective in preventing liver tumors in rats on a rice-carrot diet unless supplements of casein were fed (11). On a similar diet Nakahara *et al.* likewise failed to observe any protective effect due to riboflavin (21), presumably because their basal diet was inadequate for riboflavin retention. To the extent that casein contributes to riboflavin retention, it might be expected to retard tumor development. Nevertheless, experiments in this laboratory indicate that the incidence of tumors was essentially the same on diets containing 18 per cent (20) or 48 per cent (16) of casein, as on 12 per cent, although the latter experiment was not uncomplicated, since such high levels of protein can alter the need of the animal for vitamin B₆ (14).

In the present investigation rats were fed various azo dyes in diets containing 6, 9, 12, 24, or 36 per cent of casein or 12 per cent of casein supplemented with enough methionine to equal that in a diet containing 24 or 36 per cent of casein, and the rates of tumor formation were determined. In parallel short-time experiments rats were fed azo dyes and various levels of protein, and the storage of riboflavin in the livers was determined.

METHODS

For the production of tumors, essentially the same procedures were followed as those used in previous studies from this laboratory (18, 20); the

* Published with the approval of the director of the Wisconsin Agricultural Experiment Station. Supported in part by the Jonathan Bowman Cancer Fund and by a grant from the Committee on Growth, American Cancer Society.

† Present address: Department of Chemistry, Stanford University, Stanford, California.

‡ United States Public Health Service Predoctorate Research Fellow.

azo dyes were incorporated in the diets and fed to young adult rats for various periods of time, followed by an 8-week period on the same diet without the dye. In the initial experiments the diets fed contained 12, 24, or 36 per cent of casein, 4 per cent of Wesson salts, 5 per cent of corn oil, and glucose (cerelose) to 100 per cent. The following levels of B vitamins were incorporated into a kilogram of diet: thiamine, 3.0 mg.; pyridoxine, 2.5 mg.; calcium pantothenate, 7.0 mg.; choline, 30.0 mg.; and riboflavin, 1.0, 2.0, or 20.0 mg. For certain groups 3.6 or 7.2 gm. of DL-methionine were

given was a relatively mild one, fed in a high concentration, viz., 0.096 per cent of *o*'methyl-*p*-dimethylaminoazobenzene (*o*'Me.DAB) (Table 1, groups 7-9). In a corresponding series, 0.06 per cent of DAB was fed for 19 weeks (Table 1, groups 10-12); and the latter dye was also fed for 20 weeks in diets containing 12 per cent of casein, 12 per cent of casein plus 0.72 per cent of methionine, 24 per cent of casein, and 36 per cent of casein (Table 1, groups 13-16).

In an effort to determine the effects of low levels of protein, 0.06 per cent of DAB was fed in diets

TABLE 1
THE EFFECT OF PROTEIN OR METHIONINE ON THE DEVELOPMENT OF LIVER TUMORS
IN RATS FED CERTAIN AZO DYES

Group*	Carcinogen†	Diet	Time dye was fed (wk.)	Average initial weight (gm.)	Average at end of dye- feeding (gm.)	Average food intake (gm/rat/ day)	Survival at end of dye- feeding	Final number of tumors	Nega- tive sur- vivors	Per cent tumors
1	0.04% <i>m</i> 'Me.DAB	12% casein	18	166	199	11.8	14/14	14	0	100
2	0.04% <i>m</i> 'Me.DAB	24% casein	18	165	225	10.6	13/14	6	7	47
3	0.04% <i>m</i> 'Me.DAB	24% casein—20 mg. ribofla- vin/kg	18	168	226	11.1	14/14	1	13	7
4	0.04% <i>m</i> 'Me.DAB	12% casein	14	179	182	10.0	15/15	13	2	87
5	0.04% <i>m</i> 'Me.DAB	12% casein+3.6 gm. methi- onine/kg	14	173	203	10.3	15/15	10	5	67
6	0.04% <i>m</i> 'Me.DAB	24% casein	14	172	210	10.3	15/15	10	5	67
7	0.096% <i>o</i> 'Me.DAB	12% casein	19	178	184	8.9	14/15	11	3	79
8	0.096% <i>o</i> 'Me.DAB	12% casein+3.6 gm. methi- onine/kg	19	179	234	10.7	13/15	4	9	31
9	0.096% <i>o</i> 'Me.DAB	24% casein	19	174	224	11.0	14/15	7	7	50
10	0.06% DAB	12% casein	19	180	192	10.1	15/15	3	11	20
11	0.06% DAB	12% casein+3.6 gm. methi- onine/kg	19	182	221	10.5	15/15	3	12	20
12	0.06% DAB	24% casein	19	180	230	11.0	15/15	4	10	26
13	0.06% DAB	12% casein	20	207	254	13.9	12/15	4	7	33
14	0.06% DAB	12% casein+7.2 gm. methi- onine/kg	20	201	257	14.1	14/15	2	11	14
15	0.06% DAB	24% casein	20	203	285	15.6	12/15	2	10	17
16	0.06% DAB	36% casein	20	208	307	17.7	14/15	7	7	50

* Groups 1-2 and 4-12 received 2 mg. of riboflavin per kilogram of diet; groups 13-16 received 1 mg. of riboflavin per kilogram.

† Diet—

o' Me.DAB

added per kilogram of the 12 per cent casein diet, in order to double or triple its methionine content (casein contains 3.1 per cent of methionine [24]). Each rat received 2 drops of halibut-liver oil by dropper every 4 weeks.

In the first series (Table 1, groups 1-3) 0.04 per cent of *m*'methyl-*p*-dimethylaminoazobenzene (*m*'Me.DAB) was fed for 18 weeks to young adult rats in a 12 per cent casein diet, in a 24 per cent casein diet, and in one containing 24 per cent of casein plus a high level of riboflavin (20 mg/kg). In a second series (groups 4-6) the dye was fed for 14 weeks in diets containing 12 per cent of casein, 12 per cent of casein plus 3.6 mg. of methionine per gram, or 24 per cent of casein. In a third series these diets were fed for 19 weeks, but the carcinogen

containing 6, 9, or 12 per cent of protein and 2 or 20 mg. of riboflavin per kilogram of diet. After 3 months, however, the mortality on the lower levels of protein was great, and the condition of the survivors so poor (see also 18) that the percentage of protein in all diets was raised to 12 per cent. At the same time, the concentration of riboflavin in the low riboflavin diets was lowered to 1 mg/kg of diet in an effort to keep the diet as carcinogenic as possible. The latter diet was fed for 5 weeks, after which the diets minus the dye were fed for two additional months, when the survivors were killed for a final tumor count.

In the experiments on riboflavin retention, adult rats were fed various azo dyes in diets containing 12 or 24 per cent of casein or 12 per cent of casein

plus 4 gm. of methionine per kilogram. The concentrations of the dyes fed were 0.06 per cent DAB, 0.04 or 0.064 per cent *m'*Me.DAB, and 0.096 per cent *o'*Me.DAB. At the end of 3 or 6 weeks the animals were killed and the livers analyzed for riboflavin by the fluorometric method used in our previous studies (4, 5). The method is essentially that of Conner and Straub (2) as modified by Andrews (1).

RESULTS

Dietary protein and tumor development.—The ingestion of extra protein or of methionine invariably improved the growth and general physical condition of the rats fed azo dyes and also tended to decrease the severity of the cirrhosis that developed. The effects of the supplements on tumor formation, however, were less definite. In some

and the high protein diets, respectively (groups 7 and 9, Table 1), while in the group receiving the low protein diet plus methionine, the incidence of tumors was only 31 per cent. On the other hand, rats fed 0.06 per cent of DAB developed approximately the same number of tumors, no matter which level of protein was fed. In all groups the incidence was low, 14 to 50 per cent (groups 10-16, Table 1). A possible protective effect of protein was suggested by group 15, which received 24 per cent of casein and in which the incidence of tumors was 17 per cent, as compared to 33 per cent for a group on a lower level of casein. However, when 36 per cent of protein was fed, the incidence of tumors was highest of all, 50 per cent (Table 1, group 16).

When 0.06 per cent of DAB was fed in diets containing only low levels of protein for 3 months, fol-

TABLE 2
EFFECT OF LOW LEVELS OF PROTEIN ON PRODUCTION OF HEPATIC TUMORS IN RATS FED
0.06 PER CENT OF *p*-DIMETHYLAMINOAZOBENZENE FOR 17 WEEKS

Group	Diet*	Average initial weight (gm.)	Average	Average	Survival at end of dye-feeding	Tumors at termination of dye-feeding	Cirrhosis at termination	Negative survivors	Percent tumors
			weight at end of dye-feeding (gm.)	Food intake on dye (gm./rat/day)					
17	6-12% casein	2-1 mg. riboflavin/kg	209	19.5	11.2	7/14	2	0	5 29
18	6-12% casein	20 mg. riboflavin/kg	227	19.0	12.4	9/14	0	0	8 0
19	9-12% casein	2-1 mg. riboflavin/kg	227	22.0	12.4	9/14	0	3	6 0
20	9-12% casein	20 mg. riboflavin/kg	221	21.8	13.4	10/14	0	0	10 0
21	12% casein	2-1 mg. riboflavin/kg	201	23.3	12.6	18/15	1	0	9 8
22	12% casein	20 mg. riboflavin/kg	210	23.3	14.4	9/15	0	1	8 0

* In groups 17-20 the lower level of casein was fed during the first 12 weeks of the experiment, while 12 per cent casein was fed during the last 5 weeks of dye-feeding and during the subsequent 8 weeks on the basal dye-free diet. In groups 17, 19, and 21, 2 mg. of riboflavin/kg of diet were fed the first 12 weeks and 1 mg/kg of diet during the remainder of the experiment.

series the consumption of the high protein diet resulted in a decreased incidence of hepatic tumors, but this effect did not appear under all the various experimental conditions imposed. The most pronounced effect of protein was in a series fed 0.04 per cent of *m'*Me.DAB for 18 weeks. At the end of the experimental period, 100 per cent of the animals fed 12 per cent of casein had developed tumors, while only 47 per cent of those on 24 per cent of casein had developed tumors (Table 1, groups 1 and 2). However, in a second series in which this level of dye was fed for 14 weeks, the incidence of hepatic tumors on the low and high protein diets were 87 and 67 per cent, respectively, while the incidence of tumors in a group fed the lower level of casein plus 0.36 per cent methionine also was 67 per cent (Table 1, groups 4, 5, and 6). Protein appeared to retard tumor formation somewhat when the carcinogen was 0.096 per cent *o'*Me.DAB fed for 19 weeks; the percentages of the animals developing tumors were 79 and 50 on the low

levelled by 12 per cent of casein for 5 weeks, the incidence of tumors was low in all groups (Table 2). The highest incidence, 29 per cent, was observed in the group receiving the lowest level of protein. In line with previous experience (16, 20, 28), the chief effects of the low protein diets, however, were greater losses in weight and a generally poorer physical condition than those observed in the group receiving 12 per cent of casein throughout the experiment. The addition of riboflavin to the three diets did not affect the weights of the rats on any of the levels of dietary protein (Table 2), although riboflavin appeared to prevent the development of tumors at the lowest levels of casein fed. Its effect at higher levels could not be ascertained in this series, since no tumors developed in the control groups on 9 per cent of casein and only 1 developed in the groups on 12 per cent of casein.

Storage of hepatic riboflavin.—In line with previous observations (4, 10), the azo dyes lowered both the concentration of riboflavin in liver tissue and

the total amounts per liver (Table 3, groups 23 and 24, 26 and 28). Even in the presence of the azo dyes, casein and methionine tended to improve the retention of riboflavin, although a supplement of casein (24 per cent of the diet instead of 12) was usually more effective than an amount of methionine equivalent to that in the supplement. In a typical series fed 0.06 per cent of DAB (Table 3, groups 29, 30, 31), 17.2 µgm. of riboflavin were found per gram of liver in the group fed 12 per cent of casein, 19.6 in the group supplemented with methionine, and 20.8 in the group on 24 per cent of casein. Improved retention was also apparent

when the results were expressed as the total amount of riboflavin per liver. A similar effect of casein or of methionine was observed in series fed 0.04 per cent of *m*'Me.DAB (Table 3, groups 32–34) or 0.096 per cent of *o*'Me.DAB (Table 3, groups 35 and 36). While there were fairly wide differences in riboflavin concentration noted between individual values in the same group, the general tendency of casein to improve riboflavin retention was a consistent one among the various groups. Quantitatively, the effect of 24 per cent of casein on riboflavin retention was at least as great in this study as that observed previously when

TABLE 3
RELATIVE EFFECT OF METHIONINE AND PROTEIN ON LIVER RIBOFLAVIN IN RATS FED AZO DYES

GROUP	DIET* AND DYE FED	No. ANIMALS	INITIAL WEIGHT	GROWTH INCRE- MENT (GM/RAT/ WEEK)	DAILY FOOD INTAKE (GM/RAT/ DAY)	LIVER RIBOFLAVIN		Total µgm
						µgm/gm	Range	
23	12% casein—control	3	246	10	15.0	19.3	16.3–20.3	182
24	12% casein+0.06% DAB†	6	180	— 2	11.5	15.6	12.6–17.5	114
25	12% casein+0.06% DAB+4 gm. methionine/kg	6	181	2	11.5	18.4	16.7–20.5	145
26	12% casein+0.064% <i>m</i> 'Me.DAB	3	240	— 10	10.0	15.5	14.8–16.2	100
27	12% casein+0.064% <i>m</i> 'Me.DAB+4 gm. methionine/kg	3	215	2	11.5	15.9	15.0–17.6	108
28	12% casein, restricted control	3	249	2	11.9	20.8	19.0–21.5	162
29	12% casein+0.06% DAB	3	258	— 2	12.0	17.2	17.1–17.3	180
30	12% casein+4 gm. methionine/kg+0.06% DAB	3	250	9	14.0	19.6	15.7–22.0	184
31	24% casein+0.06% DAB	3	270	0	14.0	20.8	19.9–22.3	220
32	12% casein+0.04% <i>m</i> 'Me.DAB	3	270	— 12	10.0	14.9	13.4–16.2	109
33	12% casein+4 gm. methionine/kg+0.04% <i>m</i> 'Me.DAB	3	255	— 13	8.1	15.6	14.7–16.5	145
34	24% casein+0.04% <i>m</i> 'Me.DAB	3	270	— 2	14.0	16.8	13.8–19.2	157
35	12% casein+0.096% <i>o</i> 'Me.DAB	3	250	— 1	12.5	17.0	16.0–17.9	176
36	12% casein+0.096% <i>o</i> 'Me.DAB+4 gm. methionine/kg	3	270	— 4	13.0	19.3	16.7–23.3	200

* Diets 23–28 contained 2 mg. riboflavin/kg; diets 29–36 contained 1 mg/kg. Feeding period for all groups was 3 weeks.

† DAB = *p*-dimethylaminoazobenzene.

TABLE 4
PROTECTIVE EFFECT OF HYDROGENATED COCONUT OIL ON HEPATIC RIBOFLAVIN IN RATS FED *p*-DIMETHYLAMINOAZOBENZENE
(Data of Griffin and Baumann [5], Table III)

% DAB	Time (wk.)	Riboflavin*/gm liver (µgm.)	Riboflavin*/total liver (µgm.)
0.06	3	17.1–14.4= 2.7	101—75=26
0.09	3	18.3–15.8= 2.5	134—121=13
0.06	3	18.2–16.2= 2.0	118—113= 5
0.09	6	13.6–15.0= —1.4	125—98=28
0.09	6	17.5–17.0= 0.5	130—95=35
0.09	6	19.0–16.7= 2.3	94—75=19
Average protection due to hydrogenated coconut oil		1.4	21

* The control group received corn oil, and the third is the difference between the two.

diets were fed that are known to retard tumor development (5).

Representative data (5) are tabulated in Table 4, indicating the extent to which hydrogenated coconut oil (HCNO), an effective agent in retarding the development of tumors due to DAB (17), increases the retention of riboflavin in the liver. Under the various conditions of dosage studied, the increased retention of riboflavin in the livers from rats fed HCNO averaged 1.4 µgm. of riboflavin per gram of liver and 21 µgm. per total liver more than that in rats fed corn oil. By similar calculations of the data in Table 3, it can be shown (Table 5) that methionine is at least as effective as HCNO in improving the hepatic retention of riboflavin, 1.7 µgm. more per gram and 20 µgm. more

per liver, while protein (24 per cent instead of 12 per cent) is distinctly better than either, 2.8 μgm . more per gram and 44 μgm . more per liver (Table 5). Nevertheless, extra protein is not particularly effective in retarding the development of liver tumors (Tables 1 and 2). The results indicate that the retention of riboflavin in rats in short-time experiments is only a rough indication of the resistance of the animal to azo dye tumors and is not completely reliable under all dietary conditions.

DISCUSSION

In the present study the most obvious effect of dietary protein or methionine noted in rats exposed to azo dyes was an improvement in weight

TABLE 5

PROTECTIVE EFFECT OF PROTEIN AND METHIONINE ON HEPATIC RIBOFLAVIN IN RATS FED AZO DYES (Data from Table 3)

Group	Riboflavin*/gm. liver ($\mu\text{gm}.$)	Riboflavin*/total liver ($\mu\text{gm}.$)
<i>Protein:</i>		
0.06% DAB	20.8-17.2=3.6	220-180=40
0.04% m'Me.DAB	16.8-14.9=1.9	157-109=48
Average protection due to protein	2.8	44
<i>Methionine:</i>		
0.06% DAB, 6 wk.	18.4-15.6=2.8	145-114=31
0.06% DAB, 3 wk.	19.6-17.2=2.4	184-180=4
0.064% m'Me.DAB	15.9-15.5=0.4	108-100=8
0.04% m'Me.DAB	15.6-14.9=0.7	145-109=36
0.096% m'Me.DAB	19.3-17.0=2.3	200-176=24
Average protection due to methio- nine	1.7	20
the group receivin amount is the c	the group gure is the ; the third	

maintenance and in the general appearance of the animals. There was some indication of a slight protective effect of protein against tumor formation, but, in line with previous experiments (8, 16, 20), the effect was not marked. Tannenbaum and Silverstone have failed to find any protective action of dietary protein against spontaneous hepatomas in mice (29). The present results with protein suggest those recorded for diets high in choline, in which cirrhosis is depressed (15), the general condition of the animals improved, but the incidence of tumors is very similar to that on control diets (6, 15). It is doubtful whether the beneficial effects of dietary protein in rats fed azo dyes involve the critical carcinogenic reaction. More probably they represent merely another example of the ability of dietary protein to increase the resistance of animals to such diverse toxic agents as benzene (30), chloroform (19), arsphenamine (13),

selenium (26), and the carcinogenic hydrocarbons (32).

Dietary protein proved to be almost as effective in improving the retention of riboflavin in rats fed azo dyes as in rats not so exposed, but the significance of this vitamin in the precancerous liver is not entirely clear. Hydrogenated coconut oil, which is effective in preventing the development of liver tumors in rats fed DAB (17), is only moderately active in maintaining normal concentrations of riboflavin in the liver (5); dietary protein, on the other hand, is not particularly effective in preventing liver tumors (Table 1) but is quite active in maintaining the level of riboflavin in the liver (Tables 3 and 5). Nevertheless, hepatic riboflavin cannot be completely unrelated to the carcinogenic process, since the administration of the vitamin is an effective way of suppressing the development of hepatic tumors due to azo dyes.

In the determination of hepatic riboflavin in groups fed carcinogenic or protective diets, the variations within groups are usually fairly wide, with some overlapping of values between groups. This parallels the results of experiments in which tumors are produced, since some individuals within any group develop tumors faster than others and there is an "overlapping" of values in the sense that a few animals on a protective diet develop tumors, while a few on the carcinogenic diet do not do so. It would be interesting to know whether the individuals that maintain the higher levels of riboflavin in a short-term experiment are also the ones most resistant to tumor development.

SUMMARY

Rats fed diets containing an azo dye—DAB, o'Me.DAB, or m'Me.DAB—usually developed more hepatic tumors when the diet contained 12 per cent of casein than when 24 per cent of casein or 12 per cent of casein plus methionine were fed. The effect of the protein on body-weight and general well-being, however, was much more marked than it was on tumor incidence.

Both casein and methionine were effective in improving the hepatic retention of riboflavin by rats fed the azo dyes studied. The effect of 12 per cent of casein was greater than that of 0.4 per cent methionine.

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The Effect of Diethylstilbestrol on the Incidence of Leukemia in Male Mice of the Rockefeller Institute Leukemia Strain (R.I.L.)

JAMES B. MURPHY, M.D., AND ERNEST STURM

(From the Laboratories of the Rockefeller Institute for Medical Research, New York 21, New York)

The incidence of leukemia in the Rockefeller Institute Leukemia strain (R.I.L.) is definitely higher in the females than in the males. In a previous investigation it was shown that castrated males had an incidence as high as intact females, while castrated females treated with testosterone showed a rate closely approximating that of the normal males (4). In a group of intact males treated with theelin included in the reported study, the dosage used proved so toxic that the majority of test mice died before or in the early leukemia age period. Since the result of estrogen treatment was inconclusive, this part of the investigation has been repeated, with diethylstilbestrol instead of theelin.

EXPERIMENTAL

A group of 116 male mice from the R.I.L. strain were given weekly intramuscular injections of 0.05 cc. of diethylstilbestrol in oil (0.05 mg.), starting

TABLE 1

	No. mice	Average age at death from leukemia (wk.)	Leukemia rate (per cent)
Treated males	116	44.0	71.5
Control males	89	45.1	41.5
Control females	40	48.3	70.0

when the animals were 16 weeks of age. The injections were continued over a period of 7 months, the number of treatments varying between 21 and 28. As controls, 89 males—littermates of the test animals—were kept under the same laboratory conditions throughout the experimental period. In addition, 40 intact, untreated females were set aside to give the leukemia rate for the period of the experiment.¹

¹ During the period of inbreeding in our laboratory, the R.I.L. strain has shown a definite fluctuation in incidence of leukemia. However, a difference in incidence between males and females has been consistently maintained. For example, in 1943 the rates in females was 88.4 per cent, compared to 53.5 in males (4). In the present series the females had a rate of 70 per cent, while the males showed a rate of 41.5 per cent.

The results of the test are given in Table 1 and graphically shown in Fig. 1. It will be noted that the rate in stilbestrol-treated males was 71.5 per cent, while that for the control males was only 41.5 per cent. This rate for the treated males is almost identical with that for the control female group (70 per cent). The average age at which leukemia death occurred varied slightly between the groups. The figure for the females was 48.3 weeks; the treated males, 44 weeks; and the control males, 45 weeks.

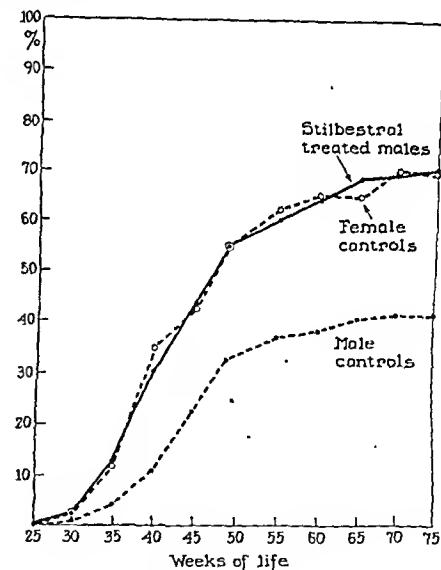


FIG. 1.—The points on the curves represent the number of mice that had developed leukemia by the age indicated, expressed as a percentage of the initial number of animals.

DISCUSSION

It was concluded in our previous communication (4) that the male sex hormone exerted some inhibitory action on the development of leukemia in the R.I.L. strain. This conclusion was based on the fact that there was a marked increase in incidence of leukemia in castrated males, and a de-

crease in incidence in females treated with testosterone. Removal of the ovaries had no effect on the rate in females. In this connection, however, it should be noted that at the time of the study the normal rate in females was so high (88.4 per cent) that further increase from removal of the ovaries might not have been possible. Gardner (1) and Lacassagne (3) have both reported that prolonged treatment with estrogenic hormones increases the leukemia rates in some strains of mice. Later, Gardner, Dougherty, and Williams (2) noted that after similar treatment the response in the increase in numbers of lymphoid tumors varies between strains, a neoplastic condition in which there is no sex difference in the rate of occurrence.

In interpreting the present result, it is difficult to say whether the estrogen acted as a stimulant to the development of leukemia or through interference with the inhibiting action in the male hormones. The fact that the rate in stilbestrol-treated males was increased no more than it was following removal of the testicles may perhaps be taken as favoring the latter interpretation.

SUMMARY

The Rockefeller Institute Leukemia strain of mice shows a consistently higher incidence of the disease in females than in males. The rate in normal males during the period of the present study was 41.5 per cent and the females 70. Prolonged treatment of male mice with stilbestrol increased the rate to 71 per cent, a close approximation to that shown by the control females. It is suggested that this result is due to the interference with the male sex hormone rather than to a direct action of the estrogen.

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The Citric Acid Content of Tumor Tissue and of Tumor-bearing Rats*

FRANCES L. HAVEN, PH.D., CHALLISS RANDALL, M.S., AND W. R. BLOOR, PH.D.

(From the Department of Biochemistry, School of Medicine and Dentistry, University of Rochester, Rochester, New York)

A high content of citric acid has been shown by Dickens (1) to be characteristic of rapidly growing and regenerating tissue. Embryonic tissue, tissue of the newborn, precancerous tissues, and a wide variety of tumors of animals and of man were found by him to be high in citric acid. Since the non-necrotic tissue of Crocker mouse sarcoma 180 contained more citric acid than did the necrotic tissue, Dickens concluded that the high citric acid content was a property of the tumor tissue itself and not the result of a change, such as calcification accompanying the necrosis.

The experiments described in this paper were undertaken to determine the citric acid content of the transplantable tumor, Walker carcinoma 256, and of various tissues of rats bearing this tumor. In some cases the whole tumor was analyzed, and in others the necrotic and non-necrotic portions of the same tumor were analyzed separately. Citric acid was determined on liver, kidney, spleen, whole blood, and blood plasma of tumor-bearing rats and was compared with values obtained on the corresponding tissues of rats in which the tumor did not grow after transplantation (non-take rats).

MATERIALS AND METHODS

Young male rats of the Wistar strain, maintained on Purina Fox Chow and water, were used throughout these experiments. Tumor tissue was transplanted subcutaneously into the groin by the trocar method. The majority of the tumors made up from 10 to 35 per cent of the total weights when the animals were anesthetized, bled, killed, and the specified tissues removed. If liver was to be analyzed, the animals were deprived of food about 18 hours previously. Blood was obtained under ether anesthesia, either from the abdominal aorta or by heart puncture. Both whole blood and plasma were extracted with 10 per cent trichloroacetic acid solution. Relative separation of tumor

tissue into necrotic and non-necrotic portions was carried out by careful dissection. All tissues were ground with sand and extracted with 10 per cent trichloroacetic acid solution until extraction was complete. Kidneys, plasma, and occasionally blood from two animals carefully matched in body-weight and tumor age and size were pooled, in order to obtain samples sufficiently large for analysis.

All citric acid determinations were made by the method of Pucher, Sherman, and Vickery (2), modified as previously reported (3). Among numerous substances tried by Pucher and others, the only substance found to enhance the color given by citric acid in this method is β -hydroxybutyric acid. In view of this fact, determinations of β -hydroxybutyric acid were carried out by Behre's (4) method on samples of necrotic and non-necrotic tumor tissue and on kidneys of tumor-bearing animals and gave colors equivalent to a maximum of only 0.2 per cent of the total amount of citric acid found. Therefore, β -hydroxybutyric acid does not contribute to the values reported as citric acid.

RESULTS AND DISCUSSION

The citric acid content of whole tumors and of non-necrotic and necrotic portions of other tumors is shown in Table 1. The values for the citric acid content of whole tumors ranged from 8.6 to 19.5 mg. per 100 gm., with a median value of 12.3 mg. per 100 gm. of fresh tissue. These values are similar to those reported by Dickens (1) for two Walker 256 tumors. Non-necrotic portions of tumor contained from 3.4 to 10.0 mg. of citric acid per 100 gm., with a median value of 5.8 mg. per 100 gm., while necrotic portions of the same tumors contained from 20.0 to 70.4 mg. of citric acid per 100 gm., with a median value of 47.5 mg. per 100 gm. The markedly higher content of citric acid in the necrotic center of the tumor than in the non-necrotic periphery is diametrically opposed to the findings of Dickens (1) on Crocker mouse sarcoma 180.

* This investigation was supported by a grant from the Donner Foundation. Presented in part at the Thirty-eighth Annual Meeting of the American Association for Cancer Research, Inc., at Chicago, May, 1947.

The citric acid content of the non-necrotic peripheral tumor tissue was inversely proportional to the size of the tumor, as shown in Fig. 1. This finding constitutes further evidence that the composition of the tumor is not always constant, a fact previously reported by Mider *et al.* (5). The relationship between the citric acid concentration in the peripheral tissue and the size of the tumor might be the result of differences in oxygen tension between small and large tumors.

Since the separation of tumor tissue into necrotic and non-necrotic portions was not absolute, the citric acid in the non-necrotic portion

forming citric acid, determinations of the activity of aconitase, the enzyme which catalyzes the conversion of cis-aconitic to citric acid, were carried out essentially by the method of Johnson (6). The fresh tissues were homogenized in the buffer, and aconitase activity was determined immediately on aliquots of the homogenate. The final concentration of cis-aconitic acid in the incubation mixture was 0.04 M, a concentration which was found sufficiently high for maximum activity of the enzyme.

Aconitase activity was present in peripheral tumor tissue, as shown in Table 2, but was not

TABLE 1
CITRIC ACID CONTENT OF WALKER TUMOR 256

No. of TUMORS	TUMOR WEIGHT (GM.)	CITRIC ACID	
		Range (mg./100 gm. wet wt.)	Median
Periphery	21	21.2-67.6	5.8
Center		{ 3.4-10.0 20.0-70.4 8.6-19.5	47.5
Whole tumor	18	13.4-62.0	12.3

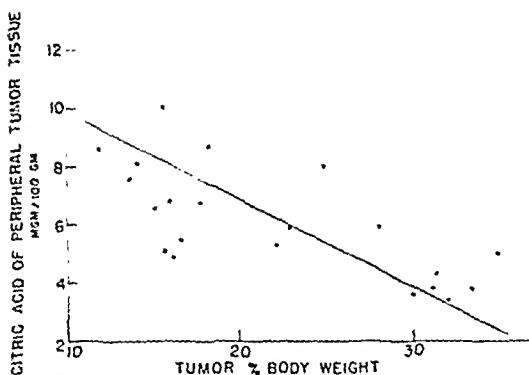


Fig. 1.—The citric acid content (mg/100 gm wet weight) of peripheral tumor tissue plotted against the tumor percentage of body-weight. The line is based on average values.

might be attributed to contamination of the sample with necrotic tissue high in citrate. This possibility is unlikely for several reasons. Citric acid was found in pooled samples of metastatic growth known to be relatively free from necrosis. Moreover, if citric acid of the periphery were due to chance contamination, it is extremely unlikely that the regular relationship shown in Fig. 1 would be obtained. Citric acid seems to be a metabolic product of actively growing tumor tissue.

The high content of citric acid in the center of the tumor might be due to (a) increased formation or decreased oxidation of citric acid within the center or (b) accumulation of citric acid formed in the periphery. In order to find out whether the tissue in the center of the tumor is capable of

TABLE 2*
THE ACONITASE ACTIVITY OF PERIPHERAL AND
CENTRAL TUMOR TISSUE

Tumor tissue	Mg. dry tissue per ml. of incubation mixture	Mg. citric acid formed per ml. of incubation mixture	$Q_{citrate}$
Peripheral	2.5	0.34	15.0
	2.1	0.31	17.2
	2.8	0.51	21.3
	2.7	0.47	22.3
	3.0	0.35	18.7
	3.6	0.51	16.1
Central	3.0	0	0
	4.8	0	0
	5.4	0	0

* Incubation mixtures were prepared by combining 3 ml. of 0.1M phosphate buffer of pH 7.4; 1 ml. of 0.2 M cis-aconitic acid neutralized in ice-cold water with solid NaHCO₃; and 1 ml. of the tissue homogenate in 0.1 M phosphate buffer of pH 7.4. Incubation was carried out for 1 hour at 40° C. under anaerobic conditions.

found in the tissue from the center of the tumor. Therefore, one can assume that citrate is not formed in the center of the tumor through the action of aconitase. Instead, the citric acid formed in the periphery may have accumulated in the center and been held there, perhaps in combination with some cation which would remove it from active metabolism. The rapid growth of the periphery and the poor blood supply of the center, which would foster anaerobic conditions, would also contribute to the accumulation of citric acid in the center.

Values for the citric acid content of organs of tumor-bearing rats and of rats in which the tumor did not take are shown in Table 3. In general, the average values for citric acid were higher in the

organs of animals with tumor. The exception presented by whole blood is not surprising, since Gathe and Nygaard (7) showed that eitrate added to whole blood remained in the plasma. On the other hand, plasma of animals with tumor was higher in citrate than was the plasma of "non-take" rats.

Two explanations can be offered for the higher eitrate content of the organs of tumor-bearing rats. The eitrate formed in the tumor in large quantities or oxidized slowly might be carried in the

3. The citric acid content of the peripheral tumor tissue was inversely proportional to the size of the tumor.

4. Plasma, liver, kidney, and spleen of rats bearing tumor were higher in eitric acid than were similar tissues of "non-take" rats.

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TABLE 3
CITRIC ACID CONTENT OF RAT TISSUES
(Mg/100 Gm Wet Weight)

Tissue	Tumor Animals				Non-Takes			
	No. of rats	No. of analyses	Mean	Range	No. of rats	No. of analyses	Mean	Range
Whole blood*	22	11	4.63	3.67- 6.54	20	10	4.98	3.43-6.53
Plasma*	25	12	6.29	4.80- 7.83	20	10	4.86	4.19-5.62
Kidney	50	21	8.88	6.46-12.40	52	19	6.11	4.05-7.64
Liver	29	29	5.97	3.51-10.47	21	21	4.41	3.06-5.72
Spleen	21	10	8.23	6.06-10.94	37	10	6.81	4.14-8.77

* Mg/100 ml.

plasma and accumulate in the liver, kidney, and spleen. Or, in line with the many observations on the chemistry of the tumor-bearing host assembled by Greenstein (8), the presence of neoplasm in the animal may have caused the various organs to assume "chemically neoplastic qualities," resulting in an increased formation or decreased utilization of citrate.

SUMMARY

1. Walker 256 rat tumors contained more citric acid in the nongrowing center than in the growing periphery.

2. Aconitase activity was present in the peripheral tissue but was completely absent from the center.

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Induction of Mammary Cancer with Methylcholanthrene*

II. Histological Similarity between Carcinogen-induced Tumors and Certain Mammary Neoplasms Occurring Spontaneously

ARTHUR KIRSCHBAUM, PH.D., M.D.

(From the Department of Anatomy, University of Minnesota Medical School, Minneapolis, Minnesota)

The onset of mammary cancer has been accelerated in female mice of the dba stock by the administration of carcinogenic hydrocarbons (4, 8, 11). When the milk-agent was excluded by foster-nursing or by hybridization (e.g., F₁ hybrid crosses between dba males and females of a second stock lacking the milk-agent), it was still possible to induce mammary cancer by administering methylcholanthrene (7, 10). In the low mammary cancer NH stock, methylcholanthrene was carcinogenic for the mammary gland (13).

Carcinogen-induced adenocarcinomas of the mouse mammary gland are characterized by a large amount of squamous epithelial metaplasia, whether the carcinogen is dissolved in benzene and applied to the skin overlying the mammary gland (8, 10), injected intravenously (7), administered intranasally in oil (8), or applied subcutaneously at sites far removed from the development of mammary cancer (6). This histologic picture could be interpreted as a specific result of the keratinizing influence of the carcinogenic hydrocarbon: keratinized masses are to be found within only 10 per cent of the adenocarcinomas arising spontaneously in stocks which possess the milk-agent and exhibit a high incidence of mammary cancer (3).

In a report comparing the histology of spontaneous and carcinogen-induced mammary cancer it was concluded that the spontaneous and induced tumors are dissimilar in both their final structure and their histogenesis (10). The spontaneous mammary tumors studied had arisen in mice possessing the milk-agent. Recently the author has studied mammary tumors arising spontaneously in hybrid crosses between females of the low mammary cancer NH strain and males of subline 212,

strain dba. The histologic structure of these tumors was found to be similar to that observed in the carcinogen-induced mammary cancers. This material is being presented, and its possible significance is discussed.

MATERIALS AND METHODS

Untreated breeding female mice of the NH stock were observed for spontaneous mammary cancer. In approximately 500 females only one mammary tumor has been seen (Fig. 1). NH mice

TABLE I
OCCURRENCE OF MAMMARY TUMORS IN HYBRID
MICE OF THE NH AND DBA-(212) STRAINS

Genetic constitution	No. of mice	No. with mammary cancer	Age of mammary cancer (months)
NH×D F ₁ *	18	3	30-34
NHD×NHD F ₂	15	3	22-30
NHD×D back-cross	22	2	22-26
Total or range	55	8	22-34

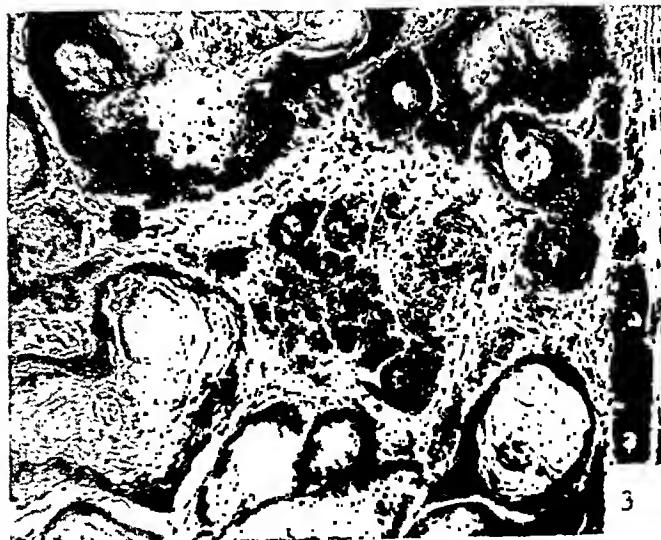
* D indicates dba-212.

were crossed with animals of the dba strain (subline 212), so that the maternal influence was always provided by the NH stock. The groups and numbers of hybrid mice studied are presented in Table I.

Hybrids all were breeding animals and were observed for the occurrence of mammary tumors. Those neoplasms which appeared were studied histologically. Comparison was made between the sections of these tumors and of mammary tumors in our collection (a) arising spontaneously in mice with the milk-agent, Strong A, dba, and (C₅H × dba) F₁ hybrids, and (b) induced by methylcholanthrene in mice with, e.g., dba-212, and with-

* This investigation has been aided by grants from the Jane Coffin Childs Memorial Fund for Medical Research, the National Cancer Institute, and the Cancer Fund of the Graduate School of the University of Minnesota.

¹ NH mice obtained from Dr. L. C. Strong, of Yale University School of Medicine, in the eighth inbred generation in 1941 and now in the twenty-fifth inbred generation.



Figs. 1 to 3

out the milk-agent, e.g., Zb mice (10) and the progeny of dba-212 mice fostered by a Zb female. Methylcholanthrene was dissolved in benzene (0.25 per cent solution) and was painted on the skin three times weekly for from eighteen to fifty times, the site of painting being varied to delay the development of skin tumors (4).

OBSERVATIONS AND DISCUSSION

The incidence and age of appearance of mammary tumors in the hybrid mice are presented in Table 1. The incidence of mammary tumors was low. This, together with the late appearance, suggests that the milk-agent is not present in the NH stock.

The typical appearance of these tumors is illustrated in Figs. 1 and 2. Histologically they are indistinguishable from methylcholanthrene-induced cancers (Fig. 3). The large amount of squamous metaplasia differentiated them from the spontaneous mammary adenocarcinomas of high cancer stocks, in which islands of keratinized epithelium are found in only a small per cent (9). Unfortunately, tumors of F_1 hybrid mice of the dba \times NH cross were not studied histologically. Such mice would be genetically identical with the F_1 hybrids studied here and would also possess the milk-agent.

Unpublished data from another laboratory also reveal that, in spontaneous mammary tumors arising in very old hybrid mice without the milk-agent, considerable amounts of squamous epithelial tissue are present (11). Other observations indicate that spontaneous tumors appearing in the absence of the milk-agent are not necessarily of this histologic type (12) and may not be essentially different histologically from tumors associated with the milk-agent (6).

The microscopic structure of mouse mammary cancer described and illustrated here (adenocarcinoma with squamous metaplasia) can be correlated with etiology other than the milk-agent (in one case carcinogenic hydrocarbon and in the other unknown). The carcinogen-induced neoplasms of the mammary gland of mice resemble certain spontaneous tumors of this species, but these are not the spontaneous adenocarcinomas usually studied and described for mice. They are tumors not ordinarily seen, since they appear in very old females of populations in which the mammary tumor incidence is low, that is, females without the milk-agent.

FIG. 1.—Mammary tumor arising spontaneously in the strain NH female. The only mammary cancer which has been seen in this stock. Large amount of squamous epithelial metaplasia. Mag. $\times 70$.

FIG. 2.—Mammary tumor arising in an F_1 hybrid female (NH \times dba). Both squamous epithelial tissue and epithelium

Studies are in progress (in collaboration with Dr. John Bittner) to ascertain whether a mammary tumor-inciting agent can be extracted from these tumors characterized histologically by squamous metaplasia.

SUMMARY

Spontaneous mammary adenocarcinomas in hybrid mice of certain genetic constitution, and presumably without the milk-agent, exhibited a pronounced degree of squamous metaplasia. The structure of these tumors was similar to that seen regularly in methylcholanthrene-induced mammary cancers. It is suggested that this histologic type of mammary tumor may be indicative of development independent of the milk-agent.

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exhibiting the acinar arrangement of mammary adenocarcinoma present. Mag. $\times 150$.

FIG. 3.—Mammary tumor induced with methylcholanthrene in a Zb female (C_5H lacking the milk-agent). Histologically, carcinogen-induced tumors are identical with mammary tumors arising spontaneously in females without the milk-agent. Mag. $\times 70$.

Studies on the Intracellular Composition of Liver and Liver Tumor from Rats Fed 4-Dimethylaminoazobenzene*

J. M. PRICE,† M.S., J. A. MILLER, PH.D., E. C. MILLER, PH.D.,
AND G. M. WEBER, B.S.

(From the McArdle Memorial Laboratory, Medical School, University of Wisconsin, Madison 6, Wisconsin)

In a previous study (8) the livers of rats fed the hepatic carcinogen, 4-dimethylaminoazobenzene, were separated by differential centrifugation into the nuclei, large granules (mitochondria), small granules (microsomes), and a supernatant fluid (particles nonsedimentable at 19,000 $\times g$). Analyses for protein, nucleic acids, riboflavin, and protein-bound aminoazo dye were made on each fraction. The ingestion of the dye in a diet low in riboflavin increased the protein and desoxypentose nucleic acid content of the nuclear fraction. In diets containing either low or high levels of riboflavin, 4-dimethylaminoazobenzene reduced the riboflavin content of the large granules and supernatant fluid, lowered the level of protein in the large granules, and decreased the pentose nucleic acid content of the large and small granules. Protein-bound dye was found in all the fractions, with the highest concentrations occurring in the small granules and supernatant fluid.

The protein-bound dyes appear to have causal significance in the carcinogenic process induced by 4-dimethylaminoazobenzene (6). Since the level of protein-bound dye in the liver reaches a maximum at 4 weeks and thereafter decreases (6), the above studies were made on rats fed the dye for this length of time. However, hepatic neoplasms do not occur until the dye has been fed for several months beyond this maximum. Therefore, it was of interest to compare the intracellular composition of the liver in rats fed the dye for 4 to 5 months and the tumors arising in such livers.

METHODS

Source and preparation of tissues.—Male rats of the Sprague-Dawley strain¹ and weighing 175 to 200 gm. were fed *ad libitum* a semisynthetic diet (9) containing 0.06 per cent 4-dimethylaminoazo-

benzene and 1.0 mg. of riboflavin per kilogram. After 4 to 5 months animals with suitable tumors were selected by exploratory laparotomy. For each fractionation of liver free of gross tumors, 2 animals were killed with ether and the livers were perfused *in situ* (8). All visible tumors were removed, and the remaining liver tissue was used for analysis. Each liver exhibited gross signs of cirrhosis due to the carcinogen.

The perfusion was omitted in the fractionations of tumor tissue, since analyses for protein-bound dye indicated that nonperfused tumors were as low in interfering blood pigments as either perfused tumors or perfused livers. This shortened the time required to collect the tumors, since 6 to 9 rats were necessary to supply enough tumor for each fractionation. After excision, each tumor was halved and inspected for signs of degeneration. All acceptable tumors were immediately placed in an ice-cold beaker, and the tumor pool was freed of external blood by washing with ice-cold isotonic KCl before homogenization. Four fractionations were made on pools of firm tumors less than 1.0 cm. in diameter. Two of these fractionations were made on tumors collected while the rats were still receiving the carcinogen, while the other two were made after the rats had been fed the same diet without 4-dimethylaminoazobenzene for an additional 2 to 4 weeks. After this period on the dye-free diet, no free (7) or protein-bound (6) dye can be detected in the liver. For comparison, one fractionation was carried out on degenerating liver tumors. These neoplasms were larger, 1 to 2 cm. in diameter, and all showed some signs of central softening, although no tumors with fluid interiors were used. All these tumors showed sufficient gross evidence of necrosis to have excluded them from the other fractionations.

Homogenates of the livers and tumors were prepared in 0.88 M sucrose solution, as described previously (8). All operations were performed in a cold room at 3° C.

Differential centrifugation.—The procedure described previously (8) was used. All centrifuga-

* This work was supported by grants from the National Cancer Institute, United States Public Health Service, and the Jane Coffin Childs Memorial Fund for Medical Research.

† Predoctorate Research Fellow, National Cancer Institute.

¹ Obtained from the Holtzman Rat Company, Madison, Wisconsin.

tions of the nuclear fraction were made at $600 \times g$ for 10 minutes (8, cf. n. 3). Three to 5.4 gm. of tumor and 7.2 gm. of liver were used for each fractionation.

Analytical procedures.—Protein, nucleic acids, riboflavin, and protein-bound dye were determined as previously described (8).

Cytological methods.—The tissues were fixed in Mossman's modification of Lavdowsky's formalin, alcohol, and acetic acid mixture (4), imbedded in paraffin, and cut at a thickness of 5 μ . Adjacent sections were stained with hematoxylin and eosin and by the Feulgen method (5). For each Feulgen-stained slide a control section was put through the same procedure, except that the acid hydrolysis was omitted.

tions compared with the whole homogenate) varied from 94 to 101 per cent (average 99).

Desoxypentose nucleic acid distribution.—On an average, the tumors contained over twice as much desoxypentose nucleic acid as did the livers, whether the data were calculated on a fresh-weight or a protein basis (Table 2). However, as a result of the large increase in the protein content of the nuclear fraction of the tumors, the concentration of desoxypentose nucleic acid in this fraction was only 114 mg. per gram of protein as compared to 91 mg. per gram for the livers. In the livers 93 to 98 per cent of the total desoxypentose nucleic acid was found in the nuclear fraction and 4 to 6 per cent in the large-granule fraction. In contrast, the nuclear fraction of the tumors contained only 82 to 91 per

TABLE 1
DISTRIBUTION OF PROTEIN IN LIVER AND TUMOR FRACTIONS
(Milligrams of Protein per Gram of Fresh Tissue; the Figures to the Nearest Whole Number)

FRACTION	LIVER DUE TO TUMORS	TUMOR CONCENTRATION					DEGENERATING TUMORS	
		During dye-feeding		After dye-feeding				
	FRACTIONATION NO.	1	2	3	4	5	6	7
Whole homogenate	139	123	122	117	121	129	123	
Nuclei	30	23	41	33	40	42	40	
Large granules	39	36	11	11	13	11	11	
Small granules	13	14	13	10	11	11	13	
Supernatant fluid	55	49	55	41	56	60	57	
Recovery	137	122	120	111	123	130	121	

RESULTS

Qualitative observations.—Unlike the relatively normal livers used in the previous study (8) and the cirrhotic livers studied here, the tumor tissue did not leave a mat of fibrous material in the tissue mincer (8) that was employed prior to homogenization. During the fractionations it was very evident that the volumes of the nuclear and large-granule fractions of the tumors were larger and smaller, respectively, than the corresponding fractions of the livers. It was also noted that the centrifuged pellet of small granules from the tumors possessed a buff color, in contrast to the amber red of the corresponding fraction from the livers.

Protein distribution.—The total protein contents of the livers and the various groups of tumors were not significantly different, but the intracellular distribution of protein changed considerably when the liver became neoplastic (Table 1). There was a sharp increase in the protein content of the nuclear fraction from an average of 27 mg. per gram of liver to an average of 41 mg. per gram of tumor, with a concomitant decrease in the average protein content of the large-granule fraction from 38 to 13 mg. of protein per gram of the respective tissues. The recovery of protein (sum of the frac-

cent of the total, with 8 to 14 per cent distributed among the three cytoplasmic fractions. The intracellular distribution of this nucleic acid was similar in both the small firm tumors and in those showing gross signs of necrosis and degeneration. It is of interest to note that, when the same fractionation procedure was applied to the livers of rats fed 4-dimethylaminoazobenzene for only 4 weeks, there was no detectable desoxypentose nucleic acid in any of the cytoplasmic fractions (8, cf. n. 3).

Cytological search for cytoplasmic particles containing desoxypentose nucleic acid.—Microscopic examination was employed to determine the origin of the desoxypentose nucleic acid in the cytoplasmic fractions. The tissues examined included sections of each of the 20 tumors used in fractionation No. 3; 7 other liver tumors which were removed with a liberal quantity of surrounding liver; and 8 liver samples from rats fed the azo dye for only 4 weeks. All the tumors examined were primary, malignant neoplasms of the liver similar to those described by Edwards and White (2). In some cases small amounts of liver were found on the periphery of tumors which had been grossly freed from liver; on the average, however, the liver cells appeared to constitute less than 5 per cent of the

tissue mass. The quantity of connective tissue varied from practically none in well-differentiated hepatomas to almost 50 per cent in some adenocarcinomas. Possibly as much as 15 per cent of the mass of a tumor pool was connective tissue.

Particularly evident in the Feulgen-stained sections of the tumors was the diffuse infiltration of polymorphonuclear leukocytes and a few round cells (Fig. 1). The tissue spaces often contained numerous leukocytes and necrotic tumor cells (Fig. 2). Small Feulgen-positive particles, which varied from about 5μ to less than 0.5μ in size, were associated with the areas containing numerous leukocytes. These particles were probably de-

tive and were much more numerous than the basophilic inclusion bodies.

Pentosenucleic acid distribution.—The increase in total pentosenucleic acid from 5.28 mg. per gram of liver to 7.03 mg. per gram of tumor was the resultant of a doubling of the amount in the nuclear and supernatant fluid fractions and a decrease in the level to one-half in the large-granule fraction (Table 3). In the large-granule fraction, however, the concentration of pentosenucleic acid per gram of protein increased from 42 mg. per gram for liver to 52 mg. per gram for tumor. On an average, 44 per cent of this nucleic acid was found in the supernatant fluid fraction

TABLE 2
DISTRIBUTION OF DESOXYPENTOSENUCLEIC ACID IN LIVER AND TUMOR FRACTIONS

FRACTION	LIVER FREED OF TUMORS	TUMORS COLLECTED					DEGENERAT- INO TUMORS
		FRACTIONATION NO.					
		1	2	3	4	5	6
MILLIGRAMS OF NUCLEIC ACID PER GRAM OF FRESH TISSUE							
Whole homogenate	2.61	2.37	5.20	6.04	5.21	5.72	5.28
Nuclei	2.57	2.20	4.54	4.95	4.73	4.73	4.57
Large granules	0.09	0.14	0.20	0.47	0.25	0.33	0.18
Small granules	0.00	0.00	0.06	0.05	0.06	0.09	0.00
Supernatant fluid	0.00	0.00	0.18	0.34	0.18	0.19	0.24
Recovery	2.70	2.34	4.98	5.81	5.22	5.34	5.08
MILLIGRAMS OF NUCLEIC ACID PER GRAM OF PROTEIN*							
Whole homogenate	19	19	43	52	43	44	43
Nuclei	86	96	111	115	118	118	114
Large granules	2	4	18	34	19	24	13
Small granules	0	0	5	5	4	6	7
Supernatant fluid	0	0	3	8	3	3	4

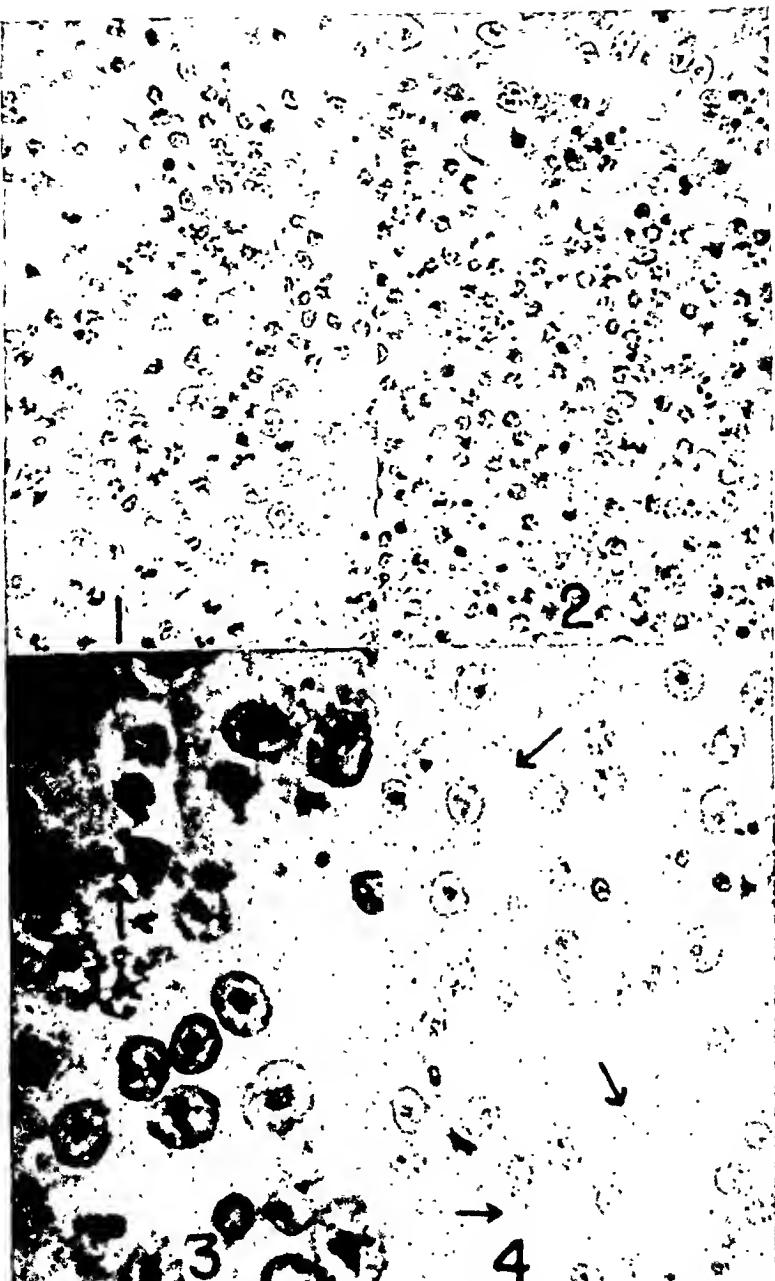
* The figures to the nearest whole number.

rived from disintegrating tumor cells and leukocytes. Occasionally these particles were found in the cytoplasm of the tumor cells, probably as a result of phagocytosis (3). Both the Feulgen-positive particles and the polymorphonuclear leukocytes were practically absent from livers of rats fed 4-dimethylaminoazobenzene for only 4 weeks and rare in livers examined after 4 months of dye-feeding. Occasionally Feulgen-positive, basophilic inclusion bodies were seen in the cytoplasm of an apparently intact tumor cell (Fig. 3). This occurred most frequently on the margin of a space containing numerous white blood cells and only occasionally in areas where there were very few leukocytes. These Feulgen-positive bodies appear to be the basophilic cytoplasmic inclusion bodies described by Edwards and White (2). Large, acidophilic cytoplasmic inclusion bodies similar to those described previously (2) were found in many of the tumors (Fig. 4). These were Feulgen-nega-

of the tumors, while the same fraction of liver contained only 28 per cent of the total. The recoveries ranged from 93 to 102 per cent (average 97).

Riboflavin distribution.—The riboflavin content of the livers and tumors averaged 5.5 and 3.9 μgm . per gram of fresh tissue, respectively (Table 4). While the riboflavin content of the large-granule fraction decreased from 2.6 μgm . per gram of liver to 1.2 μgm . per gram of tumor, the concentration per gram of protein in this fraction was 68 μgm . in the liver and 93 in the tumor. Similarly, the small-granule fraction in the tumor contained less riboflavin, but in this fraction the concentration per gram of protein was less than in the corresponding fraction of liver. On an average, the recovery of riboflavin in the fractions was 107 per cent.

Distribution of protein-bound aminoazo dye.—It was previously shown that no detectable amount of the protein-bound dyes occurs in the tumors in-



FIGS. 1 to 4.—Tissues represented by the figures on this plate were stained by the Feulgen method for desoxypentose nucleic acid. The Feulgen-positive material appears black in these photographs.

FIG. 1.—Tumor with moderate degree of infiltration with polymorphonuclear leukocytes and round cells. $\times 500$.

FIG. 2.—Same tumor, showing a large tissue space filled with leukocytes, necrotic cells, and debris. Note the small Feulgen-positive particles, some of which appear to be extra-cellular. Margin of tumor is seen at upper edge of figure. $\times 500$.

FIG. 3.—Tumor showing Feulgen-positive cytoplasmic inclusion bodies (arrow). These stained more densely and uniformly than did the nuclei. $\times 1200$.

FIG. 4.—Tumor with several Feulgen-negative cytoplasmic inclusion bodies (arrows). $\times 500$.

duced by 4-dimethylaminoazobenzene (6), and in the present studies no bound dye could be found in any of the tumor fractions. These dyes have an absorption maximum at 520 m μ in acid solution and are determined by the optical density of acid solutions of the dye after extraction from alkaline hydrolysates of the protein. Such solutions obtained from the various tumor fractions did not absorb maximally at 520 m μ , and the low optical densities found were similar to the nonspecific absorptions noted for comparable fractions from the livers of rats fed diets not containing the azo dye (8).

The liver surrounding the tumors contained protein-bound dye in all the fractions (Table 5),

but the amount of dye in each fraction was about half that found after 4 weeks of dye-feeding (8). Furthermore, in a single fractionation of the livers of rats fed the dye for only 1 week, the percentage distribution of protein-bound dye was similar to that observed after either 4 weeks or 4 months. Thus the changes in the levels of protein-bound dye apparently occur uniformly in the various fractions. Throughout the period of carcinogenesis the proteins of the supernatant fluid fraction contained the highest concentration of protein-bound dye.

DISCUSSION

Many of the changes previously found (8) in the livers of rats fed 4-dimethylaminoazobenzene for

TABLE 3
DISTRIBUTION OF PENTOSENUCLEIC ACID IN LIVER AND TUMOR FRACTIONS

FRACTION	LIVER FREE OF TUMOR	TUMORS COLLECTED					DEGENERATING TUMORS
		During dye-feeding					
		FRACTIONATION NO.					
MILLIGRAMS OF NUCLEIC ACID PER GRAM OF FRESH TISSUE							
Whole homogenate	5.27	5.17	7.03	6.13	7.59	6.88	7.50
Nuclei	0.87	0.77	1.80	1.82	1.77	1.57	1.78
Large granules	1.47	1.61	0.63	0.66	0.76	0.71	0.67
Small granules	1.08	1.33	1.24	1.09	1.26	1.32	1.58
Supernatant fluid	1.56	1.37	3.20	2.62	3.22	3.25	3.06
Recovery	4.98	5.08	6.87	6.19	7.01	6.85	7.09
MILLIGRAMS OF NUCLEIC ACID PER GRAM OF PROTEIN*							
Whole homogenate	38	42	58	52	63	53	61
Nuclei	29	33	44	42	44	37	44
Large granules	38	45	57	47	58	51	48
Small granules	83	95	95	109	90	94	122
Supernatant fluid	28	28	58	60	58	54	54

* The figures to the nearest whole number.

TABLE 4
DISTRIBUTION OF RIBOFLAVIN IN LIVER AND TUMOR FRACTIONS

FRACTION	LIVER FREE OF TUMOR	TUMORS COLLECTED					DEGENERATING TUMORS
		During dye-feeding					
		FRACTIONATION NO.					
MICROGRAMS OF RIBOFLAVIN PER GRAM OF FRESH TISSUE							
Whole homogenate	5.7	5.3	4.1	4.1	4.0	3.9	3.5
Nuclei	0.8	0.5	0.8	0.7	0.8	0.8	0.7
Large granules	2.6	2.5	1.0	1.2	1.3	1.4	1.2
Small granules	0.9	0.9	0.6	0.5	0.7	0.6	0.7
Supernatant fluid	1.7	1.2	2.0	1.8	1.6	1.5	1.3
Recovery	6.0	5.1	4.4	4.2	4.4	4.3	3.9
MICROGRAMS OF RIBOFLAVIN PER GRAM OF PROTEIN*							
Whole homogenate	41	43	34	35	33	30	28
Nuclei	27	22	20	16	20	19	18
Large granules	67	69	91	86	100	100	86
Small granules	70	64	46	50	50	43	54
Supernatant fluid	31	24	36	41	29	25	23

* The figures to the nearest whole number.

4 weeks were continued to the neoplastic state. Thus the tumors contained even higher levels of both protein and desoxypentose nucleic acid in the nuclear fraction than did the livers analyzed after either 4 weeks or 4 months. Furthermore, the large-granule fraction of the tumors contained only half as much protein, pentose nucleic acid, and riboflavin as did the same fraction of livers from rats fed the dye for only 4 weeks. On the other hand, the large granule fraction of the livers from rats fed the dye for 4 months (i.e., the liver surrounding the tumors) contained slightly more protein and pentose nucleic acid but only two-thirds as much riboflavin as was observed at 4 weeks. The

TABLE 5
DISTRIBUTION OF PROTEIN-BOUND AMINOAZO DYES
IN LIVER FREED OF TUMOR
(Measured in Acid-Ethanol at 520 $\mu\mu$ and Corrected for
Nonspecific Absorption [S])

FRACTION	$10^3 I_2/I \times 10^3$ (PER GM. FRESH TISSUE)		$10^3 I_2/I \times 10^3$ (PER GM. OF PROTEIN)	
	Fractionation No. 1	No. 2	Fractionation No. 1	No. 2
Whole homogenate	252	217	1810	1760
Nuclei	35	20	1170	870
Large granules	43	34	1100	940
Small granules	24	29	1850	2070
Supernatant fluid	149	134	2710	2740
Recovery	251	217

increased pentose nucleic acid content of the tumors and the decreased protein content of the large-granule fraction of the tumors are in agreement with the earlier findings of Schneider (10). The large amount of pentose nucleic acid in the supernatant fluid from the tumors is striking. Brachet and Jeener (1) found large quantities of this constituent in the nonsedimentable (at 200,000 $\times g$) portion of embryonic tissue and little or none in the same fractions of adult homologous tissue. Their studies did not include tumors, and further work on this point is desirable.

It is probable that the extra-nuclear desoxypentose nucleic acid found in the livers and particularly in the tumors was largely derived from fragments of the nuclei of polymorphonuclear leukocytes and necrotic tumor cells, since histologic examination of these tissues disclosed the presence of large numbers of Feulgen-positive bodies of varying size in areas where these cells were numerous. This conclusion is strengthened by the fact that, after only 4 weeks of dye-feeding, polymorphonuclear leukocytes were absent from the liver; and in this case all the desoxypentose nucleic acid was found in the nuclear fraction. In an analogous finding, Sherry *et al.* (11) noted large numbers of extra-nuclear and extra-cellular Feul-

gen-positive granules accompanied by white blood cells in sterile, as well as in purulent, pleural exudates from patients with pneumonia. It is recognized that the presence of white cells and connective tissue in the tumors studied here may be responsible in part for some of the changes in composition that were observed. The presence of some undesired tissue seems unavoidable in these fractionation studies.

While the azo dye produces considerable changes in the composition of the liver (8), the tumors which developed for 2 to 4 weeks in livers no longer subjected to the azo dye had the same composition as tumors collected during dye-feeding. Moreover, none of the fractions of the tumors collected during dye-feeding contained any detectable amount of protein-bound aminoazo dye. These aspects further emphasize the autonomous nature of the tumors.

SUMMARY

1. The livers and liver tumors of rats fed 4-dimethylaminobenzene for 4 to 5 months were homogenized and separated by differential centrifugation into the nuclei, large granules, small granules, and a supernatant fluid (particles non-sedimentable at 19,000 $\times g$). The homogenates and fractions were analyzed for protein, nucleic acids, riboflavin, and protein-bound aminoazo dyes.

2. Although the total protein contents of the livers and tumors were similar, the nuclear fraction of the tumors contained 52 per cent more, and the large granules 63 per cent less, protein than did the corresponding fractions of the livers.

3. Some desoxypentose nucleic acid was found in the large-granule fraction of the livers and in all the cytoplasmic fractions of the tumors. Histological evidence is presented that this unusual distribution of desoxypentose nucleic acid was largely due to fragments of the nuclei of white blood cells and necrotic tumor cells.

4. The nuclear and supernatant fluid fractions of the tumors contained almost twice as much pentose nucleic acid, and the large-granule fraction only half as much, as did the same fractions of the livers.

5. The large-granule fraction of the tumors contained only half as much riboflavin as did the same fraction of the livers, but the concentration of the vitamin per gram of protein increased by 38 per cent.

6. No protein-bound dye was detected in any of the tumor fractions. The percentage distribution of bound dye in the various fractions of the livers was constant from 1 week to 4 months.

7. While the composition of each fraction of the liver was altered by the ingestion of the dye, the fractions of the tumors collected either during or after feeding of the dye were nearly the same in composition.

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Studies of the Recombination of Nucleic Acids with a Tissue Polypeptide*

GEORGE T. LEWIS, PH.D., WALTER L. BLOOM, M.D.,†
AND CHARLES W. SMART, M.S.

(From the Department of Biochemistry, Emory University School of Medicine, Emory University, Georgia)

INTRODUCTION

Nucleic acids are known to occur in both plant and animal tissues, where they are thought to exist in combination with protein substances within the nucleus and in the cytoplasm. Certain of these nucleoproteins are thought to take part in the processes of cellular reproduction which are so important in the uncontrolled growth of malignant tissue. The chromosomes themselves seem to be largely made up of nucleoproteins, which thus become self-duplicating units, as was re-emphasized by Mirsky (14). However, Stedman (20) has claimed that the genetic protein is not nucleoprotein but a third substance called "chromosomin." The importance of a more complete understanding of the chemistry of these compounds is apparent to all investigators in the fields of neoplasia and cellular reproduction.

Gulland (9) in a recent review has further stressed the concept of an autosynthetic molecule as a biological unit necessary for the reproduction of cells. This review also contains a discussion of the relationship of nucleoproteins to this problem and of the nature of the nucleic acid to protein bond. Our present knowledge of the chemistry and cytology of cellular reproduction would indicate that the nucleoproteins are extremely active and necessary components in the process.

Many studies have been made on the chemical nature of the bond which presumably links the nucleic acids with the proteins in the cell. Only a few investigations have been designed to determine the chemical nature of the recombination products formed *in vitro* by nucleic acids with proteins such as those with which they might combine *in vivo*. At an early period in the investigation of nucleic acid chemistry, Milroy (13) showed that nucleic acids may combine with proteins *in vitro*. These combination products were thought

* Aided by a grant from the Georgia Division of the American Cancer Society.

† From the Departments of Biochemistry and Medicine, Emory University School of Medicine.

to behave like the naturally occurring nucleoproteins which were prepared and studied at that time. Hammersten (10) in his classical study of thymus nucleic acid in 1924 carried out the first attempt to recombine histone and nucleic acid and to study the chemical properties of the reformed nucleoprotein. Since Hammersten's work, little interest has been exhibited in extending investigations of the chemical nature of such recombination products, although such study might lead to indirect evidence concerning the linkage between nucleic acids and proteins as it exists in the cell. The general impression appears to be that this nucleic acid-protein bond is not a firm one. This is suggested by the ease with which it may be broken during manipulation incident to the isolation of nucleoprotein from tissues (17).

Greenstein (4, 5, 6) has carried out a series of investigations on the effect of the addition of proteins and of various ions on the physical characteristics of polymerized desoxyribonucleic acid. His studies indicate that the nucleic acid interacts definitely with several types of protein and with basic amino acids. According to Greenstein, such combinations result in marked alteration of physical properties of the polymerized nucleic acid isolated from animal tissues. Similar studies have also been reported by Gulland (8). However, no chemical data were obtained on the combination products of these two chemical entities (nucleic acid and protein or basic amino acids).

The present paper is concerned with a study of certain chemical properties formed by nucleic acids and a simple basic protein isolated from normal tissues. We have used the term "basic polypeptide" for this low molecular weight protein. The product, formed by combination of the basic polypeptide with polymerized desoxyribonucleic acid prepared from calf thymus, is compared with the corresponding product formed with ribonucleic acid. Definite chemical differences between the two nucleic acids have been shown (9), and, furthermore, their marked difference in respect to

polymerization is recognized; but these variations are not of sufficient degree to explain completely the presumed difference in their biological behavior. The approach described in this paper was an attempt to determine whether variations in behavior could be observed when the two types of nucleic acid were combined in the test tube with a basic tissue constituent to form a product similar to those which may exist as a nucleoprotein in the living cell.

EXPERIMENTAL

MATERIALS

A. Basic polypeptide (BPP).—This seems to be a basic low molecular weight protein which is prepared from fresh calf thymus by the method of Bloom *et al.* (1). The preparation used contained 13.49 per cent nitrogen. It was utilized in 0.5 per cent freshly prepared solutions in distilled water unless otherwise stated.

TABLE 1

EFFECT OF MIXING BPP AND DRNA

BPP (mg.)	DRNA (mg.)	Precipitate
1.25	2.5	None; viscous
1.25	1.25	Slight
1.25	0.675	Fibrous
1.25	0.337	Fibrous
1.25	0.167	Fibrous
2.5	1.25	Slight
1.25	1.25	Slight
0.675	1.25	None
0.337	1.25	None
0.167	1.25	None

B. Ribonucleic acid (RNA).—Commercial RNA (Schwarz), purified by the method of Kunitz (11), was used in 0.5 per cent solutions in distilled water. This likewise was prepared just before use. This preparation contained 13.30 per cent nitrogen and 8.01 per cent phosphorus.

C. Desoxyribonucleic acid (DRNA).—This was prepared from the nucleoprotein fraction isolated from fresh calf thymus by the method of Mirsky and Pollister (16). The protein was removed by chloroform extraction carried out according to the directions of McCarty (12). The DRNA contained 12.68 per cent nitrogen and 6.88 per cent phosphorus. Because of the high viscosity of its aqueous solutions, DRNA was used in 0.25 per cent solution in distilled water.

METHODS

In general, the method of observation of the reaction of BPP with nucleic acids was as follows: A solution of BPP was placed in a number of centrifuge tubes, and to each was added a solution of nucleic acid. The tubes were allowed to stand at 2° to 5° C. for 24 hours and then were examined

for formation of a precipitate. The physical character and approximate amount of precipitate was recorded.

If the precipitates were to be analyzed, the tubes were centrifuged and the precipitates washed twice with distilled water. The entire precipitate from each tube was then digested with 2.5 cc. of 10 N sulfuric acid for at least 2 hours. A few drops of 30 per cent hydrogen peroxide were then added, and digestion continued for a minimum of 30 minutes. Each digest was diluted to 25 cc., and aliquots were taken for phosphorus and nitrogen analyses. Usually 1-ml. aliquots were used for phosphorus determination, which was carried out according to Fiske and SubbaRow (3). Aliquots of usually 10 ml. were employed for nitrogen analysis by Wagner's boric acid modification of the micro-Kjeldahl procedure (20).

Since the ratio of nitrogen to phosphorus in nucleic acids is a fixed quantity, any increase in the ratio found upon analysis of the precipitate formed upon interaction of nucleic acid and the basic polypeptide would be due to combination with protein and, therefore, addition of protein nitrogen. Nitrogen to phosphorus ratios were thus used throughout the work to characterize the synthetic nucleoprotein obtained.

RESULTS

INTERACTION OF BPP AND DRNA

A preliminary experiment was performed to determine the effect of mixing solutions of BPP and DRNA. Two series of tubes were set up; in the first, varying amounts of DRNA solution were added to a given volume of 0.5 per cent BPP. In the second the same amount of DRNA was added to each tube, but the amounts of BPP used were varied. The results may be seen in Table 1. It is at once apparent that the weight of BPP present must equal or exceed that of DRNA in order for precipitation to occur. Analysis of a number of precipitates formed by the addition of the same amount of DRNA to a given amount of BPP showed that they contained the same proportion by weight of phosphorus and nitrogen.

An experiment was then conducted to determine whether the nitrogen/phosphorus ratio varied when increasing amounts of BPP were used with a given quantity of DRNA. The results shown in Table 2 indicate that, when more than a certain minimum of BPP is present, the nitrogen/phosphorus ratio of the precipitate formed remains constant, no matter how the relative concentration of the two materials may vary. This is also shown graphically in Fig. 1 from additional data. In other words, a stoichiometric relationship

exists between the two reactants at these ranges of concentration.

INTERACTION OF BPP AND RNA

A preliminary experiment was performed to determine the effect of BPP on RNA, as in the case of DRNA. In the first series of tubes the amount of BPP was held constant, and the quantity of RNA varied in each tube. The results are shown in Table 3. As observed above with DRNA, there must be one part or more of BPP to one of RNA for the formation of a precipitate.

TABLE 2

ANALYSIS OF PRECIPITATES RESULTING FROM MIXING BPP AND DRNA

DRNA (mg.)	BPP (mg.)	BPP/ DRNA	ANALYSIS OF PRECIPITATE		
			N (mg.)	P (mg.)	N/P
3.04	1.52	0.5	0.3542	0.1251	2.83
4.88	4.88	1.0	1.0463	0.2814	3.72
3.78	18.00	5.0	0.9287	0.2913	4.19
2.05	29.50	10.0	0.7083	0.1684	4.21
3.50	70.00	20.0	0.8925	0.1483	5.72
5.56	5.56	1.0	1.0350	0.2862	3.62
4.33	21.65	5.0	1.1274	0.2675	4.21
3.24	32.40	10.0	0.6258	0.1450	4.32
3.72	74.40	20.0	0.9571	0.2307	4.15
4.15	4.15	1.0	0.6572	0.1706	3.85
3.18	15.00	5.0	0.7048	0.1801	4.41
3.88	38.80	10.0	0.8382	0.2144	3.91
3.06	61.20	20.0	1.4147	0.3309	4.27

As with DRNA, experiments were conducted to ascertain whether or not the nitrogen/phosphorus ratio was constant at a given concentration of BPP and of RNA and also if the nitrogen/phosphorus ratio varied when the same amount of RNA was added to increasing quantities of BPP. The results, presented in Table 4, indicate not only that the nitrogen/phosphorus ratio varies at a given concentration but also that it varies widely as the relative concentrations of BPP and RNA change. These findings are shown graphically in Fig. 1, which also presents the results obtained with DRNA and BPP at the same concentrations.

DISCUSSION

The formation of a precipitate when DRNA and BPP come into contact may be taken as

TABLE 3
EFFECT OF MIXING BPP AND RNA

BPP (mg.)	RNA (mg.)	Precipitate*
5.0	5.0	Noticeable
5.0	10.0	None
5.0	20.0	None
5.0	40.0	None
5.0	5.0	Noticeable
10.0	5.0	Fair amount
20.0	5.0	Rather large
40.0	5.0	Trace

* All precipitates were particulate.

COMPOSITION OF PPT. (N/P RATIO) IN RELATION TO RELATIVE AMOUNTS OF BPP AND RNA OR DRNA

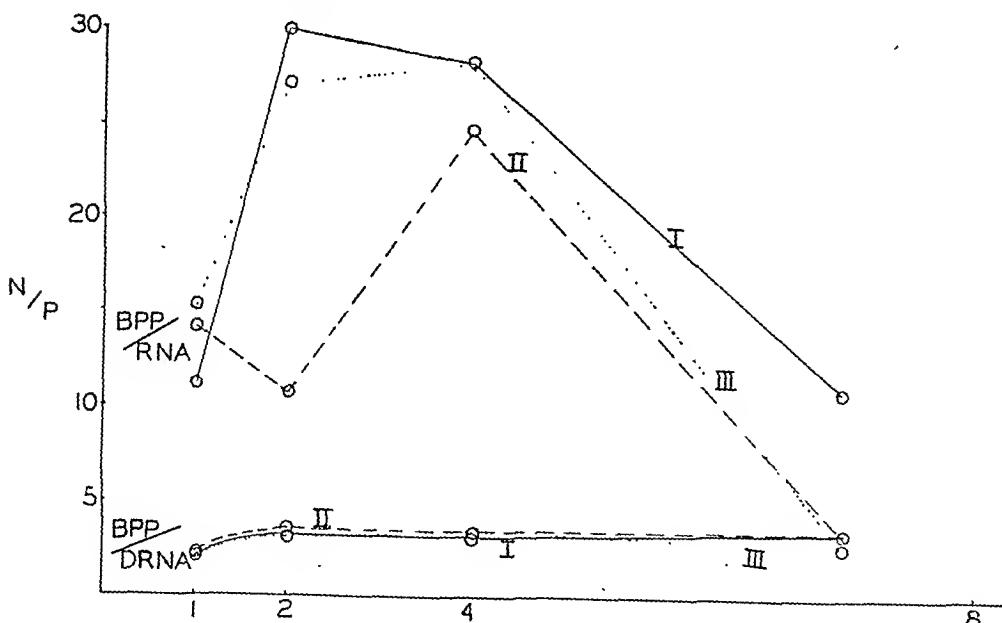


FIG. 1.—Roman numerals refer to individual experiments

indication of a physical or chemical reaction. When the two reactants are mixed in varying proportions and any precipitates formed are analyzed for nitrogen and phosphorus, the analyses show the ratio of nitrogen to phosphorus in the precipitates to be constant. This is suggestive evidence for the stoichiometric combination of the reacting substances.

This should not be surprising, since thymonucleoproteins have been known as chemical entities for some time and samples extracted under similar conditions (2, 15) give the same nitrogen/phosphorus ratio, showing that they are reproducible entities. Studies of the protein-DRNA bond in nucleoproteins have indicated that it is of the polar variety (14, 18, 19). If this is correct, re-

nucleic acids, one might anticipate the same type of combination with BPP. This, however, is not the case, as is evident from Fig. 1, which shows the reactions of the two nucleic acids to differ markedly.

While the results are not explicable on the basis of our present knowledge of nucleic acid structure, they may lead to a clue to some of the biological differences observed in the behavior of the two nucleic acids. The stoichiometric combination between the nuclear, DRNA, and basic constituent would seem likely if these components have genetic implication, as random combination of the substances would not lead to a clear concept of autosynthesis or reduplication.

From this study it would appear that the polymerization of the DRNA would be the important regulating factor in the nature of the final compound. McCarty's recent work on the influence of the DRNA of bacterial cells on the transformation of cell types would add further evidence on this point, as the DRNA appears to be one of the major controlling factors. If, as Stedman (20) has suggested, the basic protein is one of the regulating mechanisms of cellular division, the study of the basic protein-nucleic acid bond may elucidate certain of the regulatory factors concerned in the reproduction of the cell. Studies with other basic proteins, polypeptides, and amino acids may reveal further evidence which will help in the understanding of the nucleic acid-protein structure thought to be important. Furthermore, this technic may be useful in clarifying the differences in chemical behavior of the two nucleic acids, one difference having been shown in the manner in which they combine with a basic polypeptide isolated from normal tissue. This combination may possibly be similar to that in naturally occurring nucleoproteins.

SUMMARY

A precipitate is formed when solutions of BPP are mixed with solutions of RNA or of DRNA. These precipitates have been analyzed for nitrogen and for phosphorus, and the N/P ratio is found to behave characteristically for each of the nucleic acids when varying proportions of the reactants are employed. With the desoxy type the ratio remains fixed, while in the case of the ribose form it varies markedly.

This might suggest that in the cell nucleus, only one type of nucleoprotein (desoxyribonucleic acid+basic protein) is formed, regardless of the relative amounts of nucleic acid and protein present. The differences in this nucleoprotein would thus appear to depend upon the type

TABLE 4
ANALYSIS OF PRECIPITATES RESULTING FROM
MIXING BPP AND RNA

RNA (mg.)	BPP (mg.)	BPP/ RNA	ANALYSIS OF PRECIPITATE		
			N (mg.)	P (mg.)	N/P
5.0	5.0	1	1.401	0.119	11.77
5.0	10.0	2	6.894	0.270	29.97
5.0	20.0	4	7.956	0.283	28.11
5.0	40.0	8	0.921	0.084	10.96
5.0	5.0	1	1.464	0.102	14.35
5.0	10.0	2	2.833	0.262	10.81
5.0	20.0	4	8.052	0.327	24.62
5.0	40.0	8	0.236	0.076	3.11
5.0	5.0	1	1.605	0.105	15.29
5.0	10.0	2	7.083	0.262	27.03
5.0	20.0	4	7.982	0.282	28.31
5.0	40.0	8	0.188	0.070	2.68

action between the two components *in vitro* might take place by the same mechanism. The results obtained with DRNA and BPP were, therefore, not unexpected. It was anticipated that similar findings might be obtained when RNA and BPP were brought together in solution. As shown in Fig. 1, that was not the case. As the reactive mixture became richer in BPP, an increasing amount of BPP was present in the precipitate until finally a peak in the nitrogen/phosphorus ratio was reached, after which successive additions of BPP caused a decrease in the amount of BPP reacting. This phenomenon is extremely difficult to explain. The two nucleic acids, RNA and DRNA, differ in several respects: pyrimidine bases, carbohydrate, and size of molecule. However, presumably only a difference in the phosphate group would be significant in this case. Even in Gulland's postulated structure of RNA (7), three of the four phosphate groups are comparable to those found in the generally accepted structure of DRNA. Because of this similarity in structure of the two

polymer of nucleic acid and not upon the nucleic acid-protein bond. On the other hand, the corresponding combination in the cytoplasm might give rise to a series of compounds containing differing proportions of the components.

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The Effects of Hepatic and Splenic Extracts from Human Lymphomata upon the Lymphatic System of Experimental Animals

JOHN B. STORER, M.D.,* AND CLARENCE C. LUSHBAUGH, PH.D., M.D.

(From the Department of Pathology, University of Chicago, Chicago 37, Illinois)

INTRODUCTION

A theory (14, 15) has recently been formulated that leukemia and Hodgkin's disease are the result of an imbalance of normal substances which stimulate production and maturation of lymphoid and myeloid cells. This is based on the extraction of seemingly specific lymphoid- and myeloid-stimulating substances from the urine (12, 13, 14, 16, 19, 21, 23), feces (19), livers (3), and spleens (3) of human subjects with leukemia or Hodgkin's disease and from normal beef liver (20). According to this theory, there are two such substances: one, "lymphokentric acid," is responsible for stimulating the production of lymphocytes and the maturation of myelocytes; the other, "myelokentric acid," stimulates the production of myelocytes and causes lymphocytes to mature. Miller and Turner (14, 15) postulate that leukemia is due to an excess of one of these substances, which causes increased production of the involved cells without maturation, as the consequence of a relative deficiency of the other maturing substance. Similarly, Hodgkin's disease is explained as the result of an excess of both substances. The large amount of supporting data would seem to make this a valid explanation of the intermediate cause of these diseases, were it not for the fact that Heinle, Wearn, and co-workers (7, 8, 9), the parent-group originating this research, have not been able to demonstrate the presence of "lymphokentric acid" and have failed to show Hodgkin's disease-like reactions in guinea pigs injected with extracts of urine from patients with that disease. The hypothesis of Miller is untenable if no lymphokentric substance can be shown to exist. No independent studies have been made which either confirm or deny these findings or resolve the differences of the two responsible groups. Also, as Furth (4, 5) has pointed out, no information exists as to whether these substances are merely by-products of the growth of

lymphocytic and myelopoietic tissues or are etiologically responsible for the production of the diseases of these tissues.

The study reported here was undertaken in an attempt to determine the existence or absence of a chemical lymphocytic stimulator in human lymphomata and to discover whether extracts made according to the directions of Erf, Turner, and Miller (3, 20), which supposedly contain a substance etiologically responsible for leukemia, would cause this disease in susceptible laboratory animals.

EXPERIMENTAL STUDIES

Extracts were made from the livers and spleens of 11 patients who died of various lymphomata. These organs of 1 patient with erythroblastotic anemia and 1 patient with sympathoblastoma were extracted to use as controls. The methods used were those of Miller and his collaborators (3, 20), with little modification.¹ Extraction was carried to

¹ *Method I.*—The organs were ground and air-dried. They were then thoroughly extracted with 95 per cent ethanol in a Soxhlet apparatus. The ethanol extract was then saponified by refluxing for 4 hours with an equal volume of 2 N NaOH or KOH in ethanol. The solution was then diluted with water and thoroughly washed with petroleum ether (b.p. 30° to 60° C.) and the ether phase discarded. It was then acidified with concentrated HCl and extracted with petroleum ether. The ether was removed by distillation and the residue dissolved in six times its volume of acetone and cooled to -15° C. It was then filtered and the precipitate discarded. The acetone was removed by distillation and the residue further concentrated *in vacuo*. The end-product was a brown oil.

Method II.—This modification was used in order that the destruction of any biologically active substance might be minimized. The method was identical with Method I except that saponification was performed at room temperature for 1 hour rather than by refluxing for 4 hours. The end-product was a brown oil.

Method III.—The organs were ground and then mixed with five times their weight of methanol. The mixture was acidified to Congo red and allowed to stand for 3 days. It was then filtered and the residue discarded. The methanol solution was partially concentrated *in vacuo*, diluted with water, and extracted with petroleum ether. The ether phase was washed first with water and then 2 N NaOH. The ether phase was dis-

* Postgraduate Fellow of the United States Public Health Service, 1948-1949.

the point where this group was first able to show strong lymphocytic-stimulating activity in urinary extracts (19). The assays of these materials did not warrant further extraction and purification. The pertinent data concerning these extracts are summarized in Table 1.

The extracts were tested for biological activity by the method of Erf, Turner, and Miller (3). Thirty-seven guinea pigs, averaging 400 gm. in weight, were injected subcutaneously with a single dose of 0.8 to 6.3 ml. of the extracts, representing from 135 to 310 gm. of unextracted organ. Except where the yield was low or the toxicity of the extract high, the dose was equivalent to 250 gm. of whole liver or spleen. Most of the extracts elicited

made of the liver, spleen, lymph nodes, thymus, lungs, adrenal gland, kidneys, and bone marrow of all animals and stained with hematoxylin and eosin and Wright's stain.

Histologically, using the guinea pigs injected with mineral oil as the most comparable controls, no evidence of definite specific lymphocytic stimulation could be found. Without these animals for comparison, the animals injected with the extracts showed slight activity by the criteria of Erf, Turner, and Miller (3). This evaluation is summarized in Table 2, along with the doses and organ equivalents of the extracts. The lungs were found to be of no value in this assay, since the majority of the guinea pigs, regardless of previous treatment,

TABLE 1
THE SOURCES AND YIELDS OF THE EXTRACTED MATERIAL

Extract no.	Pathological diagnosis	Extraction method*	Organ	Wet weight (gm.)	Extract yield (ml.)
1	Aleukemic lymphatic leukemia	I	Liver	2830	56.0
			Spleen	925	4.0
2	Hodgkin's disease	I	Liver	3400	51.0
			Spleen	1450	4.0
3	Chronic lymphatic leukemia	II	Liver	3210	24.7
			Spleen	2490	26.0
4	Acute stem-cell leukemia	I	Spleen	205	3.9
5	Lymphosarcoma	II	Liver	2020	45.2
			Spleen	710	1.5
6	Polycythemia rubra vera terminating in lymphatic leukemia	II	Liver	2580	34.3
			Spleen	590	3.5
7	Chronic lymphatic leukemia	I	Liver	1350	18.6
			Spleen	150	2.0
8	Lymphosarcoma	I	Liver	1805	23.0
9	Lymphoblastic leukemia	III	Liver	765	0.3
10	Stem-cell leukemia	III	Liver	2400	1.8
			Spleen	620	0.1
11	Lymphosarcoma	III	Liver	1630	2.9
12	Erythroblastic (Cooley's) anemia	I	Liver	2550	23.0
			Liver	2900	0.1
13	Sympatheticoblastoma	III	Spleen	210	0.1

* See n. 1, p. 108.

a severe local foreign-body reaction but did not prove particularly toxic. Only extract 6 from the liver of the patient with polycythemia rubra vera and lymphatic leukemia killed any of the guinea pigs. The remainder of the animals were sacrificed 28 to 32 days following the injection and did not appear ill at that time. Because all the extracts appeared to be irritant oils, 9 guinea pigs were given comparable-sized subcutaneous injections of light mineral oil. Four untreated guinea pigs were killed, and their organs, along with those of many other guinea pigs used for other purposes in the laboratory, were used as further controls. Sections were

carried. The alkaline solution was then acidified and extracted with petroleum ether. The ether was evaporated *in vacuo*; the residue was dissolved in acetone and cooled to -15° C. It was then filtered and the precipitate discarded. The acetone was removed by distillation, and the material was further concentrated *in vacuo*. The end-product was a brown oil.

showed large nodular infiltrates of mature and immature lymphocytes around the pulmonary blood vessels. Many untreated animals showed even more extensive infiltrates than did the treated ones. Lymphocytic infiltrates in the liver were larger and more numerous in the animals receiving the extracts than in the untreated animals, but they were only slightly larger than those in the livers of the guinea pigs receiving mineral oil. The infiltrates consisted predominantly of mature small lymphocytes, regardless of the original source of the extract. The extract from the case of Hodgkin's disease did not cause a Hodgkin's disease-like infiltrate. The largest lymphocytic infiltrates were present in the kidneys. The most extensive of these infiltrates in this organ were found in the guinea pigs injected with mineral oil. No foci of immature lymphocytes were found. Megakaryocytes were not seen in the spleens of the un-

treated animals but were found in the animals injected with mineral oil or the extracts. They were most numerous with the latter treatment. The splenic lymphoid follicles were hyperplastic in all the animals. In the animals injected with the extracts these follicles did not exceed in size, number, or mitotic activity those of the animals with mineral oil. The lymph nodes were also hyperplastic in all the animals. In general, the adrenal glands showed little change: Rare foci of extra-medullary myelopoiesis and perivascular lymphocytes were seen in them. There were no changes in the thymus glands or the bone marrow.

Since animals susceptible to lymphatic leukemia might be more sensitive to a stimulator of lymphocytes, these extracts were also injected into strain Ak mice.² For this purpose 24 mice were used,

stimulator which these extracts are said to contain is leukemogenic, 100 strain Ak mice were injected with these extracts as a preliminary experiment. The mice averaged 2 months of age at the time of injection. Although some of the material oozed from the injection site when doses greater than 0.5 ml. were given, autopsy of mice dying shortly after injection showed the brown oil present throughout the connective tissue of the back or, in some instances, localized in foreign-body granulomas. Sloughs were not commonly obtained, but in no instance where the mice survived longer than 180 days was oil actually demonstrated in the fibrous tissue at the original site of injection. Because of the acute toxicity of these extracts for mice and the fighting habit of this strain, only 47 of the injected mice survived longer than 180 days. In this experi-

TABLE 2
BIOASSAY OF ACTIVITY OF THE EXTRACTS

Source of extract	No. of cases extracted	No. of animals used in assay	Dosage range (ml.)	Organ equivalent (gm.)	Grade of reaction*
Lymphatic leukemia	3	15 guinea pigs	1.5-3.8	150-275	2-3
		14 Ak mice	0.06-0.5	14-32	0
Lymphosarcoma	2	8 guinea pigs	3.3-6.3	260-280	2-3
		6 Ak mice	0.16-3.1	11-189	0
Stem-cell leukemia	1	4 Ak mice	0.25-0.5	13-26	0
		4 guinea pigs	0.8-4.1	270-290	1-2
Hodgkin's disease	1	5 Ak mice	0.04-0.2	13-14.5	0
		6 guinea pigs	1.8-3.6	135-370	0-1
Polycythemia rubra vera with lymphatic leukemia	1	4 Ak mice	0.09-0.25	13-42	0
		4 guinea pigs	2.8	310	2
Cooley's anemia	1	1 Ak mouse	0.25	28	0
		9 guinea pigs	0.5-4.0	Untreated	2-3
Mineral oil	-	4 guinea pigs	Untreated	Untreated	0-1
		20 Ak mice	Untreated	Untreated	0

* Degree of hyperplasia and cellular infiltration of the organs of the injected animals was graded according to the methods of Erf, Turner, and Miller (3): 0 = no reaction, 1 = minimal reaction, 2 = moderate reaction, 3 = maximal reaction.

along with 10 additional mice that died during the first several weeks following the injection of the extracts, to observe chronic effects. The mice were sacrificed 29 to 39 days after injection and studied histologically for any evidence of leukemic change or lymphocytic infiltrates. Twenty mice were used as uninjected controls. In spite of the fact that these mice received, on the basis of body-weight, about two to three times the dose of extract that the guinea pigs received, none of the organs of these animals injected with any of the extracts showed any lymphocytic infiltrates in excess of the minute foci normally seen in these animals (see Table 2).

In order to determine whether or not these extracts might contain any leukemogenic substances or, on the other hand, whether the lymphocytic

ment 87 mice were adequately autopsied at death, and the cause of death was determined. No mouse with or without the injection died of leukemia before 180 days. For this reason, only mice living 180 days or more are included in Table 3, which summarizes the data obtained in this experiment. The average age at time of death from leukemia in the injected mice was 270 days for the females and 300 for the males. The ages for both sexes ranged from 192 to 418 days. The average age at death from leukemia in the 86 control mice was 286 days for females and 322 for males. The ages ranged for both sexes from 192 to 580 days. While the number of mice used in this experiment is too small for statistical significance, the similarity between the ages at death from leukemia, the ranges of the ages, and the total incidence of leukemia in the two groups when sex is disregarded indicates that the injection of these extracts had no effect upon the incidence of leukemia or the leukemic disease of these mice.

² The original stock of our colony was obtained from Dr. Jacob Furth. Inbreeding has been strictly maintained in the ensuing 3 years.

COMMENT

In these experiments no specific stimulatory effect upon the lymphatic system of guinea pigs or mice was found in hepatic or splenic extracts from cases of human lymphomata. Injection of these extracts produced in guinea pigs lymphocytic infiltrates which appeared comparable to those illustrated in the published reports (3, 14) on this subject; but bland mineral oil and the extract from the case of erythroblastic anemia also produced such infiltrates (Figs. 1 to 6). While such infiltrates are not commonly found in healthy young guinea pigs, they are caused by a wide variety of nonspecific stimuli, such as mild indigenous laboratory infec-

changed histologically by the injected material. These observations appear to indicate that these extracts did not contain leukemogens, since such substances decrease the latent period and usually increase the incidence of the disease (1, 2, 10, 17). No local tumors were found at the site of injection, but this could not be expected, since none of the injected material was found there after 180 days (18).

SUMMARY

Extracts of the livers and spleen of 8 patients dying from various lymphomata and 1 patient dying from erythroblastic anemia were tested for lymphocytic stimulation in guinea pigs and in

TABLE 3
THE EFFECT OF ORGAN EXTRACTS ON SPONTANEOUS LEUKEMIA IN AK MICE

Extract no.	Source of extract	Organ	Dose (ml.)	Organ equiv. (gm.)	Mice living over 180 days		No. dying from leukemia	Av. age at death from leukemia (days)	Range (days)	Incidence of leukemia*	
					Sex	days					
2	Hodgkin's disease	Liver	0.2	13	F	3	2	294	277-310	2	
					M	2	1	238			
3	Lymphatic leukemia	Spleen	0.5	181	F	3	1	282			
					F	6	4	284	233-310	4	
4	Stem-cell leukemia	Liver	0.25	32	M	2	0				
					F	2	1	242		1	
5	Lymphosarcoma	Spleen	0.25	23	F	5	5	282	238-376		
					F	4	3	238	192-330	4	
6	Polyeythema vera and lymphatic leukemia	Spleen	0.25	13	M	1	1	277			
					F	3	3	255	220-322	2	
7	Lymphatic leukemia	Liver	0.25	18	M	8	7	307	252-418		
					F	5	4	286	192-580	4	
8	Lymphosarcoma	Liver	0.20	20	M	8	5	306	239-361		
					F	26	19	270	192-376	1	
Totals					M	21	14	300	238-418		
Controls (uninjected)					F	53	44	286	192-580		
					M	33	15	322	219-428	0	

* The numerator indicates the number of mice dying of leukemia and the denominator the number living more than 180 days.

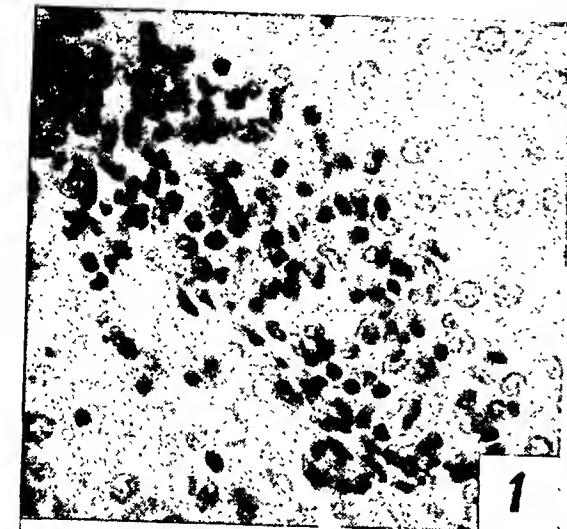
tions and injections with foreign substances. The lymphocytic foci found in strain Ak mice injected with the extracts did not exceed those seen in the normal controls, indicating for this species a lack of specific or nonspecific effect of these extracts. Only recently, Miller (11) has stated that his lymphocytic-stimulating material has never given so strong a reaction as has the myelogenous material. Heinle (6) has stated that re-examination of the material on the basis of which Wearn, Miller, and Heinle (23) first reported a specific lymphatic-stimulating factor has left him unconvinced that any significant lymphoid hyperplasia occurred.

These extracts did not hasten the onset of leukemia in the strain Ak mice. Although too few mice were used for statistical accuracy, the incidence of this disease in these animals does not appear to have been changed by the injections. When the sex of the mice is disregarded, about 70 per cent of the injected and control groups developed the disease. The character of the leukemia was not

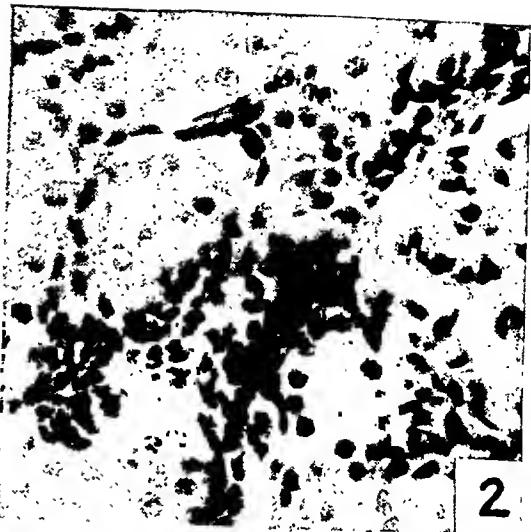
strain Ak mice. No specific lymphoid or Hodgkin's disease-like reactions were demonstrated. Non-specific reactions in the guinea pigs, similar to those previously reported, were found with the extracts from the lymphomatous cases, with the extract from erythroblastic anemia, and with commercial mineral oil. No lymphocytic reactions were found in the strain Ak mice, and the incidence of and time of death from leukemia in this strain were not apparently affected, although the number of animals used in this experiment was small.

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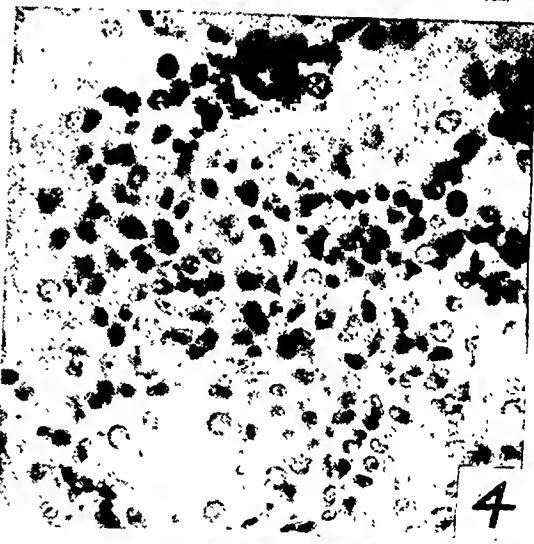
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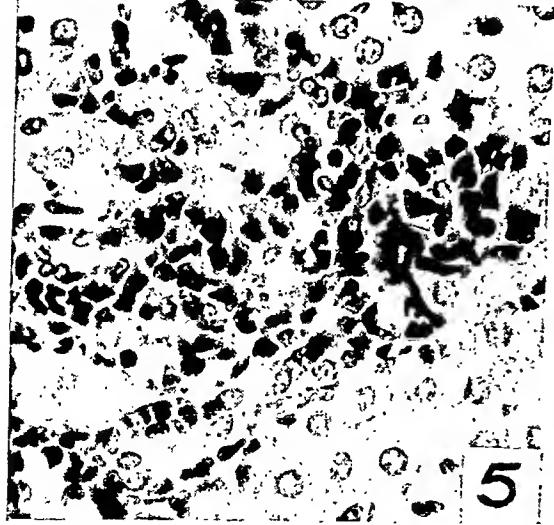
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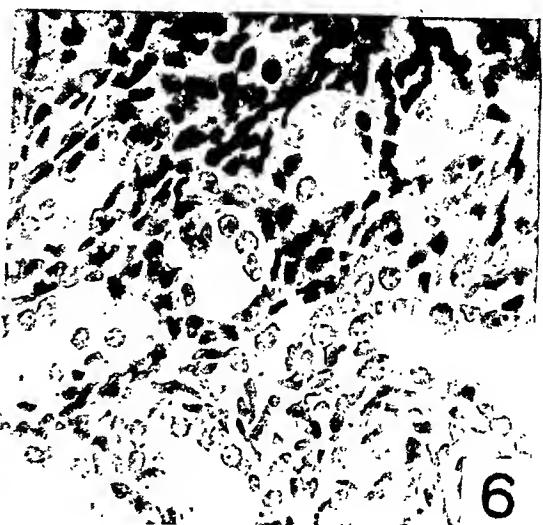
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FIGS. 1 TO 6

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Figs. 1 to 6.—Photomicrographs and sections made by Charles Blinn, technician, University of Chicago. Sections stained with hematoxylin and eosin. Magnification is approximately $\times 600$.

FIG. 1.—Infiltrate in liver of guinea pig killed 30 days after injection of 2.8 ml. of hepatic extract from case of erythroblastotic anemia.

FIG. 2.—Infiltrate in kidney of same animal.

FIG. 3.—Infiltrate in liver of guinea pig killed 30 days after

injection of 4.1 ml. of hepatic extract from case of Hodgkin's disease.

FIG. 4.—Infiltrate in kidney of guinea pig killed 30 days after injection of 3.8 ml. of hepatic extract from case of lymphatic leukemia.

FIG. 5.—Infiltrate in liver of guinea pig killed 28 days after injection of 1.0 ml. of commercial mineral oil.

FIG. 6.—Infiltrate in kidney of same animal.

The Influence of Normal and Cancer Blood on Tyrosinase Activity *in vitro**

WALTER MARX†

(From the William G. Kerekhoff Laboratories of the Biological Sciences, California Institute of Technology, Pasadena, and the Emery Tumor Clinic, Los Angeles, California)

Hirshfeld *et al.* reported that a factor in the blood serum of cancer patients inhibited the aerobic oxidation of tyrosine by crude potato tyrosinase (1). It was suggested that this inhibition of the enzyme tyrosinase might form the basis for a serological test for the diagnosis of cancer. The present report deals with an attempt to confirm these findings and to improve the reliability of the method.

BLOOD SAMPLES

Both human blood and rabbits' blood were used. Many different types of human cancer were examined (Table 1). The data reported include 130

mals having an experimentally transplanted malignant growth, either a Brown-Pearce (2) or a V-2 tumor (3);² 13 rabbits' cancer blood samples and 21 normals were tested.

EXPERIMENTS USING HIRSHFELD'S TECHNIC

A number of normal and cancer blood samples was examined, following Hirshfeld's procedure (1) as closely as possible. The rate of the oxidation was followed colorimetrically, and the color increment

TABLE I
SUMMARY OF MALIGNANT TUMOR CASES
USED FOR BLOOD SAMPLES

Origin of tumor	No. of cases
Sex organs	52
Epithelium	18
Respiratory system	15
Gastrointestinal tract	12
Skeletal and hematopoietic system	11
Miscellaneous	22
Total	130

cases in which malignancy was demonstrated by pathological examination of biopsy or post mortem material and which had had neither surgery nor radiation prior to the withdrawal of the blood sample.¹ The normal human blood samples were obtained from 209 adults of both sexes, from healthy individuals as well as from patients having various diseases but no known malignancy. The rabbits' cancer blood was derived from ani-

* Supported by a grant from the Emery Cancer Research Fund.

† Present address: Department of Biochemistry and Nutrition, University of Southern California, Los Angeles 7, California.

¹ The cancer blood samples were obtained from the clinics of the Emery Tumor Group and the Cedars of Lebanon Hospital; most of the normal blood samples were donated by the staff of the Emery Tumor Clinic. I am very much obliged to Doctors C. K. Emery, S. Hirshfeld, and R. D. Chier and to the staff of the Emery Tumor Clinic for their co-operation.

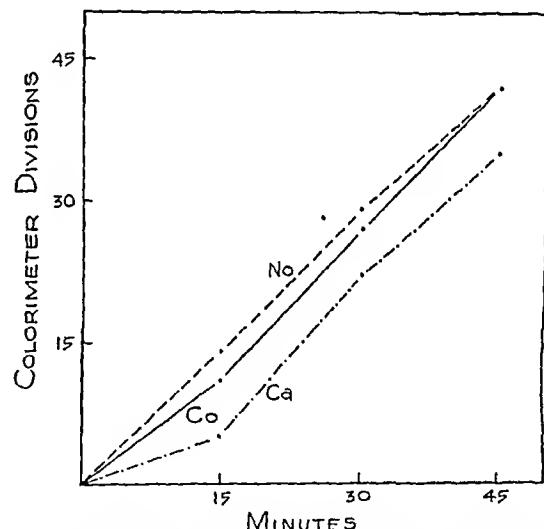


FIG. 1.—Effects of normal and cancer serum on tyrosinase activity. Experimental conditions as described by Hirshfeld *et al.* (1). No, normal serum. Ca, cancer serum. Co, control (no serum).

(I) observed after periods of 15 or 30 minutes was used as a measure of the rate of the reaction. The degree of inhibition was expressed, as a matter of convenience, by means of $(I_{Nor} - I_{Ca})$, the difference between the rates of normal and cancer blood samples.

² I am much indebted to Dr. John G. Kidd, of Cornell University, and to the National Cancer Institute for the animals carrying the tumors, and to Dr. A. L. Cohen, of this Department, for several tumor transplantations.

The results of a single test in which a normal and a cancer blood sample were compared, are illustrated in Fig. 1. In this particular experiment a relatively slight, but definite, inhibition was observed, in agreement with Hirshfeld's observations. When the whole series of experiments was considered, however, the phenomenon was not consistently demonstrable (Table 2). Two further series of tests were made under identical conditions, except that the rate of oxidation was followed for only 15 minutes. Human serum was used in the second series and rabbits' serum in the third. Table 3 summarizes the results of all three groups of experiments.

TABLE 2
EFFECT OF HUMAN NORMAL AND CANCER SERUM ON TYROSINASE ACTIVITY*

RATE (COLOR INCREMENT IN 30 MINUTES)		DIFFERENCE IN $I_{Nor} - I_{Ca}$
I_{Nor}	I_{Ca}	RATES $I_{Nor} - I_{Ca}$
32.5	{26.0 28.5	6.5 4.0
42.5	42.0	0.5
38.5	37.0	1.5
31.7	32.4	- 0.7
35.0	31.4	3.6
32.0	31.0	1.0
41.1	40.5	0.6
41.0	44.0	- 3.0
42.2	41.4	0.8
12.0	{15.0 10.0	- 3.0 2.0
45.8	37.2	8.6
25.6	{18.6 18.6	7.0 7.0
50.0	{46.0 47.0	4.0 3.0
43.0	27.5	15.5
30.0	24.0	6.0
20.0	20.0	0.0
67.0	{59.0 53.0	8.0 14.0
7.0	8.0	- 1.0
Mean difference 3.7 ± 0.9		

* Experimental conditions essentially as described by Hirshfeld *et al.* (1).

The average values of ($I_{Nor} - I_{Ca}$) indicate that tyrosine usually was oxidized at a slightly slower rate in the presence of cancer serum, but only in the first series was the mean difference statistically significant ($P < 0.01$). In many individual tests, however, the inhibition was found to be either very small or entirely lacking. In some cases the cancer serum even caused stimulation instead of inhibition. It is obvious that, under these experimental conditions, the variations were too great and the effects too small to permit use of the tyrosinase system for a clinical test. The results indicated a slight trend, however, which could be interpreted as being due to a real phenomenon, perhaps obscured by interfering factors of unknown nature.

Experimental conditions were varied, therefore, in an attempt to obtain more consistent results. The variations concerned: (1) the physical conditions, such as temperature, light, shaking, etc.; (2) the enzyme preparation; (3) the substrate; (4) the reaction medium (buffers, inorganic ions); and (5) the blood sample. Each set of experimental conditions was tested in a separate series of experiments.

PHYSICAL MODIFICATIONS OF THE PROCEDURE

Warburg manometer.—Attempts at following the rate of the reaction with a Warburg manometer failed, since the oxygen uptake of the enzyme blank (crude potato juice alone) was relatively much too great, in contradistinction to earlier findings (4).

TABLE 3
EFFECT OF NORMAL AND CANCER SERUM ON TYROSINASE ACTIVITY*

SPECIES OF SERUM SAMPLE	NUMBER OF SAMPLES (NIN.)	DURA- TION OF EXPERI- MENT	MEAN DIFFERENCE IN RATES ($I_{Nor} - I_{Ca}$) AND STANDARD ERROR			<i>P</i>
			Normal	Cancer	STANDARD ERROR	
Human	30	19	23	3.7 ± 0.9		< 0.01
Human	15	10	9	5.0 ± 3.0		0.1
Rabbit	15	13	12	0.3 ± 1.4		0.8

* Experimental conditions essentially as described by Hirshfeld *et al.* (1).

Lighting conditions.—Comparisons were made of the effects of different lighting conditions, using fluorescent light as previously described (1), incandescent light, and no special light at all. No effect was demonstrable, as far as the distinction between normal and cancer serum was concerned. No artificial light was used, therefore, in later series of experiments.

Temperature.—The test tubes containing the reaction mixture were, at first, kept in a water bath at room temperature (without thermostat). The changes occurring during the period of a single test (15 or 30 minutes) remained within 0.2° to 0.5° C. These variations did not influence the results appreciably, especially since the emphasis was placed on a comparison of normal and cancer blood samples tested simultaneously. In later series, tests were run without a water bath, without any apparent disturbing effects.

Shaking.—It was considered important to stir or shake the reaction mixtures. After several attempts with mechanical shaking devices which proved unsatisfactory, the samples were mixed before each colorimeter reading by vertical stirring

with a glass rod provided with a bead at the lower end.

Incubation period.—In a series of experiments, enzyme solution and serum were incubated for periods of 1 to 8 minutes before the substrate was added. Although, at first, such incubation appeared to increase in some instances the difference between rates of normal and cancer samples, further results were disappointing, and the incubation technic was abandoned.

MODIFICATIONS CONCERNING THE ENZYME PREPARATION

Whole potato juice.—It was soon realized that the enzyme preparation described by Hirshfeld and co-workers was highly unstable, especially when exposed to air. With a fresh enzyme solution prepared as quickly as possible, tyrosine was usually oxidized at an almost constant rate during

highly unstable. It was to be expected that, by means of purification of the enzyme, the interfering substance (or substances) would be removed and a preparation of greater stability obtained. Furthermore, various investigators emphasized that much purer, more stable, and more active preparations of the enzyme tyrosinase could be obtained from mushrooms than from potatoes (5, 6).

A white cultivated variety of the common mushroom, *Agaricus campestris* (7), was therefore used as starting material; the enzyme was precipitated by approximately 4 volumes of acetone at 2° to 4° C., and the precipitate redissolved in a small volume of saline or water. The solution of the partially purified mushroom enzyme was yellow or grayish brown, and hardly any darkening was observed during anaerobic storage at 2° to 4° C. The kinetics showed, in agreement with earlier findings (5), an induction period of about 5 to 15

TABLE 4
EFFECT OF HUMAN NORMAL AND CANCER BLOOD ON TYROSINASE ACTIVITY
(Partially Purified Enzyme Preparations)

GROUP	BLOOD SAMPLE	ENZYME PREPARATION	ACTIVATOR	BUFFER	NUMBER OF SAMPLES		MEAN DIFFERENCE IN RATES $(I_{Nor} - I_{Ca})$ AND STANDARD ERROR	P
					Normal	Cancer		
1	Plasma	Mushroom	—	Phosphate	9	9	-0.4 ± 1.4	0.8
2	Plasma	Potato	—	Veronal	18	20	2.2 ± 0.8	0.01
3	Serum	Potato	—	Veronal	12	16	0.5 ± 1.0	0.6
4	Plasma	Potato	+	Phosphate	12	13	0.3 ± 1.1	0.8
5	Plasma	Potato	+	Veronal	21	28	2.6 ± 0.9	<0.01
6	Serum	Potato	+	Veronal	10	14	0.6 ± 1.1	0.6
7	Filtrate, CCl_3CO_2H precipitation	Potato*	—	Phosphate	23	22	1.7 ± 0.8	0.05

* In a part of this series, a lyophilized preparation of whole potato juice was used.

the initial 30 minutes of the test. But when the preparation of the enzyme took a little more time or when the enzyme solution was exposed to air for a few minutes before it was used, an induction period was always observed.

It is evident that this lack of stability of the enzyme preparation was one of the factors responsible for the great variability of the results. Another cause was the fact that a new potato had to be used daily as starting material for the enzyme preparation and that enzyme preparations made from different potatoes varied greatly in their activities and kinetic characteristics, even if the same variety of potatoes was used.

Partially purified mushroom enzyme.—Obviously, in order to obtain uniform results, a more stable enzyme preparation had to be used. The possibility was considered that the observed variability of the enzyme kinetics was caused by another component (or other components) of the potato juice which influenced the enzymatic tyrosine oxidation and which, at the same time, was itself

minutes' duration, followed by an approximately constant rate for at least 45 minutes. The enzyme activity per milligram of solids and the stability of the mushroom preparations were much greater than those of the crude potato enzyme. The presence of serum or plasma modified the kinetics of the partially purified enzyme appreciably. The induction period was usually obliterated, and an initial stimulation was observed instead.

A number of tests was made, comparing normal and cancer serum and using partially purified mushroom enzyme. In some of the tests plasma was used instead of serum; otherwise conditions were essentially as described above. The results are shown in Table 4, group 1. No significant difference was demonstrable between normal and cancer blood. In spite of the fact that a purer enzyme preparation with far greater activity and stability was used, the slight trend which had been observed for the difference between normal and cancer serum in the first series of experiments

using crude potato juice, was completely obliterated under these conditions.

Partially purified potato enzyme.—Since with crude potato juice enzyme at least a trend had been noted, it was decided to make partially purified enzyme preparations from potatoes and examine their properties regarding a distinction between normal and cancer blood. Potato juice was prepared as described (1); the enzyme was precipitated by 4 to 4.5 volumes of acetone at 2° to 4° C., and the precipitate was redissolved in a small volume of distilled water or saline. The color of a fresh solution of partially purified potato enzyme was light brown and darkened only slightly during anaerobic storage. The kinetics were similar to those of the partially purified mushroom enzyme; upon exposure to air, however, darkening occurred somewhat more rapidly than in the case of the latter. The activity was greater than that of the crude potato enzyme but not as great as that of the partially purified mushroom preparation. The presence of normal serum or plasma modified the kinetics, eliminating the induction period and causing an initial stimulation instead, as was the case with partially purified mushroom enzyme.

Two series of experiments were made with partially purified potato enzyme, one using serum, another using plasma; except for the enzyme preparation, experimental conditions were essentially as described above. In the series with plasma (Table 4, group 2), the mean difference ($I_{Nor} - I_{Ca}$) was statistically significant, but its value was small (8 per cent of the mean rate for normal plasma). In a number of individual cases of this group, however, no inhibition—in several instances, even a stimulation—was found as an effect of the cancer blood. With serum (Table 4, group 3) no significant difference was observed between normal and cancer samples.

Activator from potato juice.—At this point the following working hypothesis was taken into consideration. It was postulated that, during purification of the enzyme, a substance was removed which was necessary for the inhibition by cancer serum, as observed in some of the experiments using fresh whole potato juice. It was further assumed that this substance, which was designated "activator," was unstable in the crude potato juice but stable after separation from the enzyme(s). On the chance that the activator was not a protein, it was decided to attempt separation of the activator from the enzyme, to keep both stored separately, and to recombine them only at onset of the test.

For this purpose, potato juice was prepared and

the enzyme precipitated with acetone as described above. The supernatant acetone extract was concentrated *in vacuo* under N₂, until free from acetone (water-bath temperature gradually raised to 65° C.). The aqueous residue contained a yellow pigment which had precipitated during the concentration and which gave the solution a greenish-yellow tinge; it was not further examined regarding its chemical properties. After separation from the enzyme, the activator was found to be stable for many weeks when stored in the cold; thus it was now possible to prepare and store stable stock solutions of both activator and enzyme which,

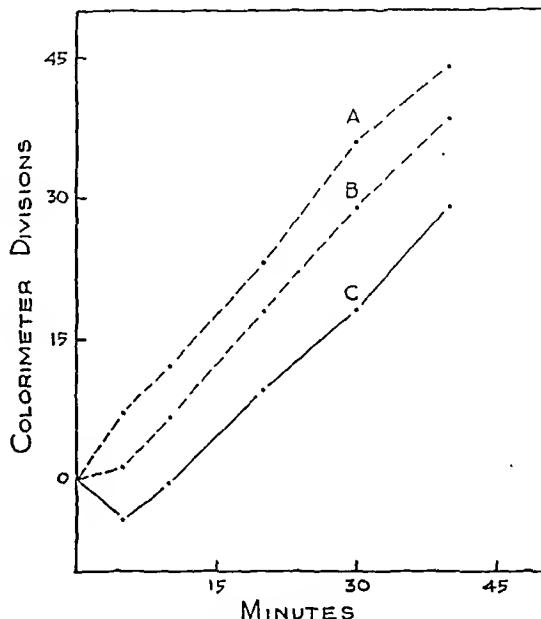


FIG. 2.—Effects of acetone extract of potato ("activator") on the activity of partially purified potato tyrosinase. A, 0.05 ml. of acetone extract. B, 0.02 ml. of acetone extract. C, no acetone extract. All three samples contained partially purified potato enzyme, tyrosine, and buffer.

when mixed in certain proportions, gave rise to well-defined and easily reproducible kinetic characteristics.

Graded amounts of the activator were added to a constant quantity of a partially purified potato-enzyme preparation. The results, shown in Fig. 2, indicate that the activator, depending on its concentration, either decreased or completely eliminated the induction period of the tyrosine oxidation.

In the following experiments a system was employed consisting of buffer, tyrosine, activator, serum or plasma, and enzyme. In order to determine optimum conditions for the distinction between normal and cancer blood, preliminary tests were made in which the activator concentra-

tions were varied, with the other components constant. The optimum concentration of activator was assumed to be that which just eliminated the induction period in the control sample.

A number of blood samples was examined under these conditions. The optimum concentrations of enzyme and activator were determined daily in preliminary control tests. Otherwise, conditions were essentially as described above. The results (shown in Table 4, groups 4 to 6) led to the following conclusions: (1) Any given single experiment may or may not show a difference between normal and cancer blood samples. (2) In the series in which plasma and veronal buffer were used (group 5), the average difference between normal and cancer samples was small (11 per cent of the mean rate for normal plasma) but statistically significant; in a number of individual experiments of this group, however, cancer plasma failed to inhibit the reaction, as compared to normal plasma. (3) In the other two series the mean difference ($I_{Nor} - I_{Ca}$) was not statistically significant.

Further purified potato enzyme.—For a further purification of the enzyme, potato juice was fractionated with acetone and the precipitate, obtained between 33 and 60 volume per cent of acetone, was further purified by ammonium sulfate fractionation; the precipitate collected between 0.4 and 0.7 saturated ammonium sulfate was dialyzed, and the undialyzable fraction was centrifuged. The supernatant fluid contained a more highly purified enzyme preparation, was lighter yellow in color, and did not darken appreciably during storage.

A number of blood tests was made, using this purer tyrosinase preparation and activator (7 normal and 7 cancer samples). The results were not more consistent than they had been in previous groups. The mean value for ($I_{Nor} - I_{Ca}$) was found to be 1.6 ± 1.2 ($P = 0.2$; statistically not significant). Obviously, under these conditions the tyrosinase system was not suitable for a clinical test to distinguish between normal and cancer blood, in spite of the fact that the enzyme preparations used were more stable and better defined than in former experiments.

MODIFICATION CONCERNING THE SUBSTRATE

The substrate concentration was varied between approximately $M/1000$ and $M/5000$; the lower concentration appeared preferable for a distinction between normal and cancer samples. The results of experiments using tyrosine as substrate were, as a whole, disappointing.

The possibility was considered that the kinetics of the oxidation of the phenol group of the tyrosine molecule were complicated by the amino group and that perhaps the presence of the latter contributed to the variability of the results. Further tests, therefore, were made using N-free substrates—in particular, phenol and *p*-cresol. In these experiments both crude potato juice and partially purified mushroom enzyme were used.

In the absence of serum an induction period was always observed with phenol and *p*-cresol as substrate. The oxidation of the former was somewhat more rapid, that of *p*-cresol very much faster, than that of tyrosine, in agreement with reports in the literature (8, 9). In order to obtain comparable reaction rates, the enzyme concentrations were adjusted to correspondingly lower levels. The products of the oxidation of phenol were red, those of *p*-cresol yellow, and neither of these solutions became brown or black in the end, as was the case with tyrosine. In the presence of normal serum or plasma the kinetics of the reaction with phenol or *p*-cresol as substrates were appreciably different from those using tyrosine and were also more complex, since both the induction period and the rate of the reaction proper were modified.

A number of experiments was made comparing normal and cancer serum, with phenol or *p*-cresol as substrates. The results again were disappointing, showing even more variability than those in which tyrosine had been used. No correlation between reaction rate and malignancy was demonstrable.

MODIFICATIONS CONCERNING INORGANIC SALTS

Following a suggestion of Dr. D. Appleman, a small amount of powdered CaCO_3 was added to the freshly prepared enzyme solutions, and, after a period of approximately 10 to 20 minutes, the excess of CaCO_3 was removed by centrifugation. This treatment had primarily the following two results: (a) The enzyme was significantly activated, so that less enzyme solution was needed for the tests, and (b) the pH of enzyme solutions treated in this way was much closer to the point of neutrality, at which the tests were usually run. But, as far as the differentiation of normal and cancer bloods was concerned, the treatment of the enzyme solution with CaCO_3 had no significant effect.

The pH of the test solution was kept near 7.0. Both phosphate and veronal buffers were used at varying concentrations. Another series of experiments was made in the absence of buffer, the pH being adjusted with NaOH and HCl . In general,

the reaction rate seemed to be little influenced by the anions used. Of the cations tested, Na^+ appeared to inhibit, but K^+ and Ca^{++} to stimulate, the oxidation of tyrosine. This observation is in agreement with findings made by Dr. Appleman (10). Regarding the distinction of normal and cancer samples, veronal buffer was superior to phosphate buffer; considering the groups summarized in Table 4, the only two series with a statistically significant mean difference in rates were those in which veronal buffer and plasma were used (groups 2 and 5). But the results were not consistent enough for a clinical test.

MODIFICATIONS CONCERNING THE BLOOD SAMPLE

Comparison of serum and plasma.—The effects of serum and plasma on the tyrosinase system were compared, using partially purified potato enzyme. Plasma was obtained from heparinized blood.³ The results shown in Table 4, groups 5 and 6, indicate that with plasma the mean difference between the rates of normal and cancer samples was statistically significant but relatively small (11 per cent of the mean rate for normal plasma), while with serum no significant effect was observed. But even in the plasma group, a number of cancer samples failed to inhibit the reaction as compared to normal plasma, as mentioned above.

Effects of deproteinized blood filtrates.—It was not known which plasma fraction contained the substance(s) responsible for the phenomenon. On the chance that the principle in question was not a protein, the protein fraction was removed by precipitation and the deproteinized filtrate tested for its effects on the tyrosine oxidation. Two different procedures were used for deproteinization; in the first series, 3.5 volumes of "acid tungstate" (11) were added to 1 volume of plasma or serum; in the second series, whole blood was deproteinized by means of adding 4 volumes of 10 per cent trichloracetic acid. In each case the precipitates were removed by filtration, and the filtrates were neutralized and added to the test solution under the conditions outlined above. Partially purified potato enzyme was used, with tyrosine as substrate.

A small number of tests was made, using tungstic acid filtrates; the results were disappointing. A larger series of blood samples was tested, using trichloracetic acid filtrates of whole blood.

³ Since commercial heparin solutions contain preservatives which interfere with the test (e.g., phenol), a powdered heparin preparation free of preservatives was used, for which I am much obliged to Dr. B. J. Brent, of Roche-Organon.

The value for $(I_{\text{Nor}} - I_{\text{Ca}})$ was again small (6 per cent of the mean rate for normal samples) and statistically not significant (Table 4, group 7).

Effect of red cells.—Since, in several instances, the serum samples showed an appreciable degree of hemolysis, the influence of hemolyzed red cells alone was investigated. Red cells were obtained from heparinized samples of both human and rabbits' blood, washed with saline, and then hemolyzed by dilution with distilled water. For the tests, varying amounts of the clear solution were added to the tyrosinase system. The results of these experiments may be summarized as follows: (1) The effects of hemolyzed red cells from normal blood samples were not significantly different from those of cancer blood samples. (2) The effects of hemolyzed human red cells were similar to those of hemolyzed red cells derived from rabbits' blood. The influence on the kinetics of the reaction was complex and varied greatly with the nature of the enzyme preparation and substrate used.

CONCLUSIONS AND SUMMARY

The effects of normal and cancer blood on the aerobic oxidation of tyrosine in the presence of tyrosinase were investigated as a test of malignancy, as recently suggested by Hirshfeld *et al.* (1). No unequivocal distinction between normal and malignant sera was possible under the experimental conditions proposed by these investigators. The reason for the disagreement between their results and those reported above is not known. Stadie *et al.* recently also reported a series of experiments in which the effects of normal and cancer sera on the tyrosinase system were studied, precisely following Hirshfeld's procedure (12). Essentially in agreement with our findings, these authors observed no correlation between malignancy and inhibition of the tyrosinase activity. When, however, in the results reported above, the average values of larger groups of experiments were considered, a small but statistically significant difference between the effects of normal and cancer sera was observed in one of three series, and a slight, if not significant, trend in the same direction was noted in a second group. In view of these observations, attempts were made to improve the reliability of the method by varying experimental conditions.

The experimental variations concerned: (1) the physical conditions, such as temperature, light, shaking, etc.; (2) the enzyme preparation; (3) the substrate; (4) the reaction medium (buffers, inorganic salts); (5) the blood sample. An unequivocal distinction between normal and cancer

blood was not possible under any of the experimental conditions examined, although appreciably purer, more stable, and more active enzyme preparations were used.

When average values for larger groups of tests were considered, however, a trend in the direction suggested by Hirshfeld *et al.* was noted in several series. This trend was very small, and it was statistically significant in only 2 of 7 groups, i.e., when plasma, partially purified potato enzyme, and veronal buffer were used with tyrosine as substrate.

It is not known whether the small trend observed in some of the experimental groups is an artifact or whether it is due to a real phenomenon obscured by interfering factors of an unknown nature.

ACKNOWLEDGMENTS

I am very much obliged to Dr. Clyde K. Emery for his interest and for a grant which made this work possible, and to Dr. Henry Borsook for his interest and guidance, for many valuable suggestions and constructive criticisms. I am also indebted to the late Dr. Samuel Hirshfeld for his enthusiasm and encouragement and to Dr. David Appleman for valuable suggestions.

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Splenic Lymphosarcomas in Rats Bearing Intrasplenic Implants of Butter Yellow*

ELIZABETH LOWENHAUPT, M.D.

(From the Division of Pathology, University of California Hospital, San Francisco 22, California)

Hepatic cellular changes produced by oral administration of *p*-dimethylaminoazobenzene (butter yellow) associated with variations of other dietary constituents have been extensively studied of late (3, 9, 15, 16, 21, 22, 23, 24, 25), and recent investigations indicate that neoplasms so induced are related to hepatic cellular changes in enzyme activity (12). Although liver cells are concerned primarily in the metabolism of butter yellow and related compounds (15), they alone with dietary administration are exposed to high concentrations of it. Thus it seems of interest to report abnormal growth in cells of another tissue exposed over long periods to high concentrations of *p*-dimethylaminoazobenzene.

MATERIALS AND METHODS

Thirty-eight rats (total from 40 surviving into the experimental period), weighing at the start 130 to 170 gm., form the basis of this report. Twenty-eight of these received pellets of butter yellow in the spleen, using the technic first developed by Mark and Biskind (19).¹ These pellets weighed between 17 and 27 mg. at the start, most in the neighborhood of 20 mg. Following operation, these 28 animals, together with 10 controls of similar weights but without splenic implants, were placed on the following diet: casein 10 per cent, sucrose 75 per cent, vegetable oil 8 per cent, salt mixture 4 per cent, cod-liver oil 2 per cent, and wheat-germ oil 1 per cent. To each kilogram of diet the following supplements were added: thiamine chloride, inositol, and paraminobenzoic acid, 40 mg. of each; riboflavin, pyridoxine hydrochloride, and calcium pantothenate, 20 mg. of each; nicotinic acid 50 mg.; and choline chloride 500 mg. Butter yellow (0.6 gm. per kilogram of diet), dissolved by gentle heat in the vegetable oil, was added to the diet given to half of the pellet-bearing animals and half of the controls.²

* Aided by a grant from Christine Breon Fund for Medical Research.

¹ The author is indebted to Dr. G. R. Biskind, Mount Zion Hospital, San Francisco, for preparation of these pellets by compression.

Ten animals in all were sacrificed at intervals up to 345 days, when 6 were killed. Three more were killed up to 461 days, when the remaining 19 were sacrificed. For the most part, the weight of the animals had continued to rise up to about 400 days, when a plateau at 450 to 550 gm. had become evident and persisted to the end of the experiment.

RESULTS

In the liver, only two tumors were observed, both at 461 days and both tiny cholangiofibromas (24). At this time nuclear changes of the type described by Opie (25) were observed in hepatic cells. However, no hepatomas occurred. This negative finding will be discussed below.

Changes associated with a pellet of oil-soluble material in the spleen can be divided into four groups, the distinction between groups 2 and 3 being quite arbitrary and that between 3 and 4 being dependent on remote organ changes. Only the first group is shown to be due directly to this foreign material. However, none of the control animals showed splenic changes at all suggestive of the other groups, and it seems justified to assume this relationship, even though the chronology of these changes is not strictly sequential. The groups are as follows.

Group 1.—A foreign-body reaction to the substance of the pellet was noted. In some spleens there apparently was considerable necrosis, with a dense fibrous wall about the area, in which there was much calcium deposited. This reaction did not seem to be associated with further changes, and the foreign material was not found in the spleen beyond the capsule itself. This is shown in Fig. 1. In other cases the reaction was of a more progressive type. That is, even after many months the process seemed to be extending, and no

² This addition to the diet was made as part of another experiment. However, since no hepatic tumors occurred to limit the duration of the experiment and since the splenic changes occurred independently of the dietary butter yellow, apparently representing a local reaction, these two groups are not further considered—the two groups considered being those with splenic implants and the controls without.

dense capsule was formed. In these cases there was an epithelioid and mononuclear cell response which formed distinct tubercles, identical histologically with the proliferative tubercles of tuberculosis. Here giant cells were formed, histologically of the Langhans type, and they were surrounded by epithelioid cells and a few fibroblasts. This sort of tissue formed a rim about the pellet, but the material did not seem to be confined to this area. Subsidiary tubercles were found removed from the chief pellet mass, and, extending through the wall of the primary mass and out into these tributary tubercles, mononuclear cells were found containing granules of yellow-brown pigment, presumably butter yellow. Some

shown in Fig. 2. The identity of these cells is discussed by Krumbhaar (18) and will be considered below.

Group 3.—Distinct nodules, presumably neoplastic, of this perifollicular tissue, with sharp borders from surrounding normal spleen, were seen in three instances. The distinction between this and the preceding group, to be sure, is quite arbitrary and is made on the basis of invasion and replacement of lymphoid follicles by this tissue, so that only scattered foci of mature lymphocytes remain to mark the site of the former follicle. The cells and the appearance of the nodules in the spleen were identical with one tumor of group 4, which metastasized. One of these spleens was approximately



FIG. 1.—Changes classified as group 1. These are direct mesenchymal reactions to the oil-soluble material of the pellet.

FIG. 1a.—Fibrotic reaction at 345 days with spread of butter yellow beyond the connective tissue capsule. $\times 160$. H and E stain.

FIG. 1b.—Proliferative granulomatous response at 40 days,

of this, often in considerable amount, was found in the near-by tissue. These features are shown in Fig. 1.

Group 2.—A second group of changes, seen in the neighborhood of pellets producing the diffuse reaction described in group 1, consisted of a hyperplasia (or at least an increase in width of this zone) of cells in the perifollicular envelope (18) (Fig. 2). It might be thought that this represented the non-specific change seen, for example, with *Salmonella* infections in the rat (8). However, there was none of the focal hemorrhage and necrosis seen with that disease, and the spleens were not grossly enlarged. Also, in many instances this change was restricted to the area of the pellet. As the process became quite extensive, wide zones of this tissue coalesced to obliterate the red pulp in this area and to surround lymphoid tissue of the follicle areas. This is

extending from a connective tissue capsule (*above*) into the surrounding spleen. $\times 80$. H and E stain.

FIG. 1c.—The same reaction showing the epithelioid nature of the cells. $\times 160$. H and E stain.

FIG. 1d.—A "tubercle" at 461 days found beyond the limits of the capsule about the pellet. $\times 320$. H and E stain.

one-third enlarged and was found at 461 days; the other two grossly within usual limits were found at 365 and 461 days. These are seen in Figs. 4 and 5.

Group 4.—Two metastasizing splenic sarcomas occurred—one found at 446 days, the other at 461 days. The former spleen was not distinctly enlarged, microscopically resembling group 3. Scattered minute metastases, however, were found in the liver. This is shown in Figs. 6, 7, and 8. The other spleen weighed 27 gm. (usual weight about 2 gm.), and tumor tissue extended from the spleen into surrounding structures. Lymph nodes were diffusely enlarged, and these, as well as liver, kidney, and skeletal muscle (diaphragm), contained tumor masses. This is shown in Figs. 9, 10, and 11.

The cells of these two tumors, as well as those of the previous groups, are essentially similar. They are large, 20 to 30 μ in diameter, with rather

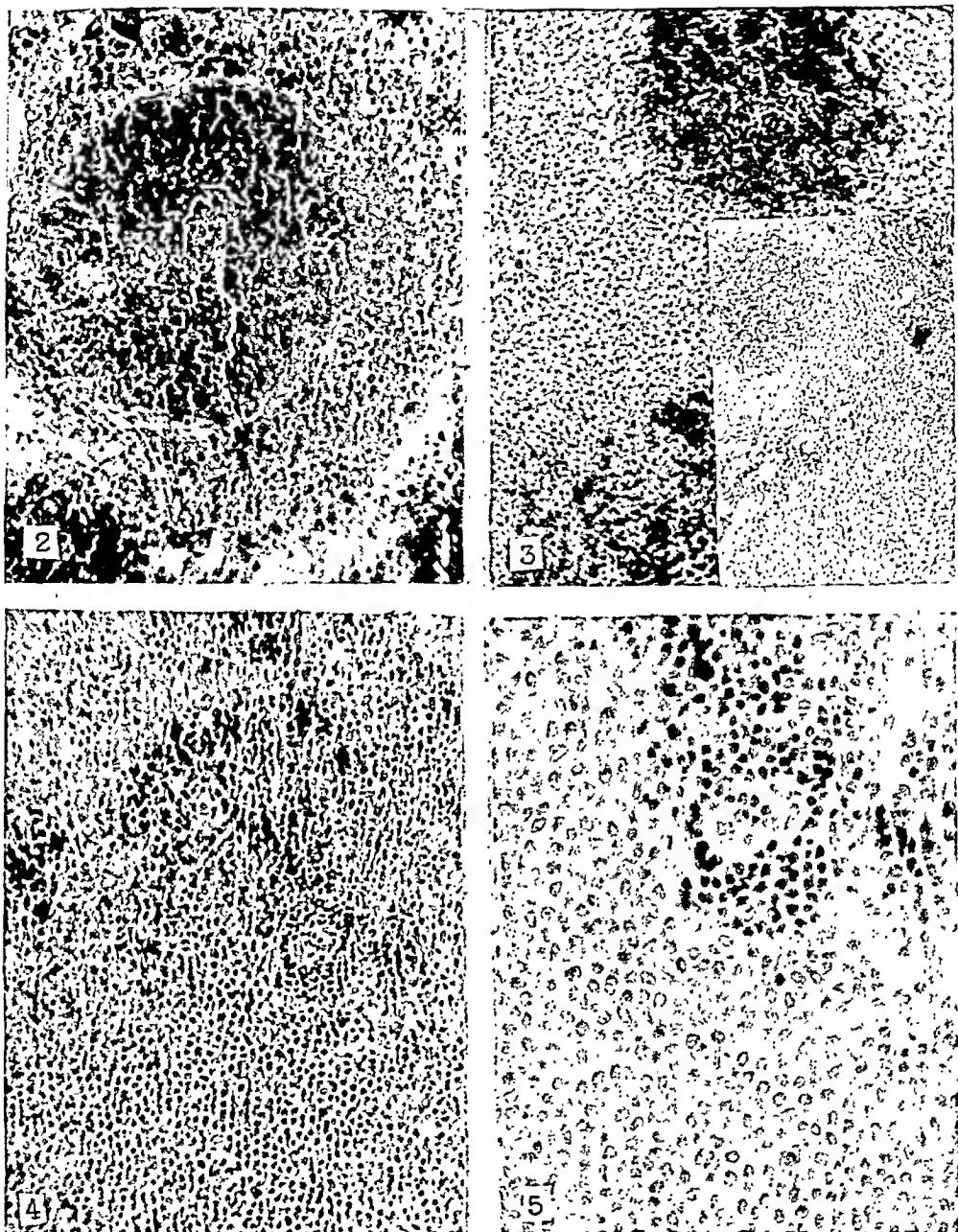
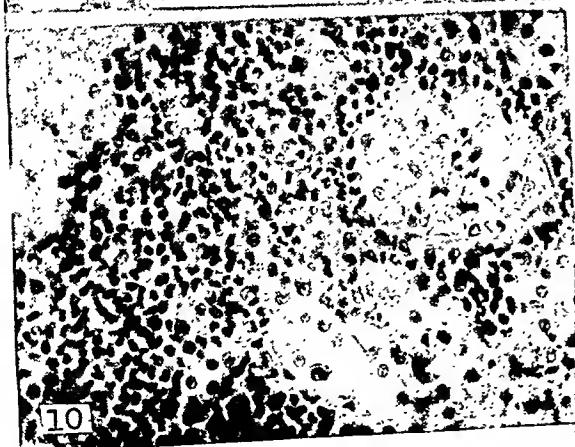
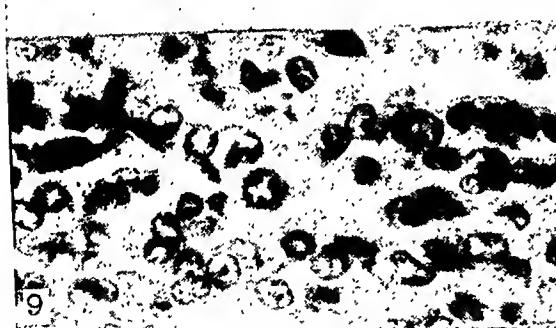
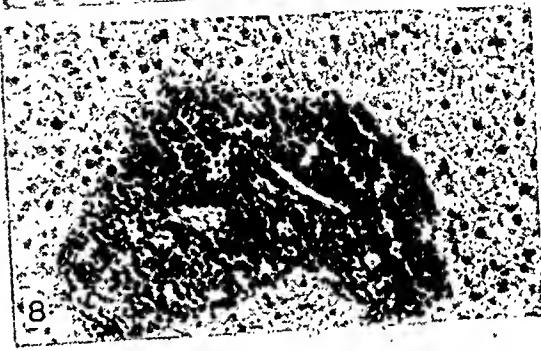
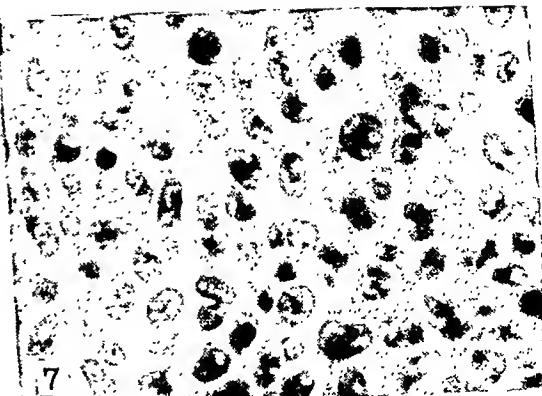


FIG. 2.—Spleen of a control rat with a Malpighian corpuscle surrounded by a narrow perifollicular zone, well defined from the surrounding red pulp and from the lymphoid tissue of the corpuscle. $\times 215$. H and E stain.

FIG. 3.—Changes in the spleen classed as group 2, seen here at 365 days. Wide, coalescing areas of cells, resembling those of the perifollicular zone, replace these zones and replace the red pulp. They surround intact lymphoid centers. $\times 160$. H and E stain. *Inset:* The same spleen to show the extent of this tissue. $\times 80$.

FIG. 4.—Changes classed as group 3, 461 days. The whole field is formed by these large, pale-staining cells, which extend irregularly into the borders of lymphoid centers, remnants of which are seen in the central area. $\times 215$. H and E stain.

FIG. 5.—Group 3 changes, again at 461 days (second spleen). This shows a remnant of a Malpighian corpuscle and its central arteriole, invaded irregularly by proliferating cells. The tissue shown here and in Fig. 4 is considered neoplastic, since it shows intrasplenic invasion and since the cells are identical with those of the tumors shown below, which formed remote metastases. $\times 430$. H and E stain.



FIGS. 6 TO 11

seant to moderately abundant cytoplasm. This is uniform, rounded, and does not contain granules with Giemsa staining; nor are phagocytized particles demonstrated in it. The nuclei are quite large, with a fairly prominent nuclear membrane. Most are round, some are indented or folded to form two coarse lobes. Chromatin is in delicate to fairly heavy bars. Each nucleus has from one to three large and prominent nucleoli. Mitotic figures are rather frequently seen. These cell characteristics are shown in Figs. 7 and 9.

DISCUSSION

Spontaneous lymphoid tumors of the spleen are not recorded in a large series of rat neoplasms (2, 4) and have not been observed in this present colony of Long-Evans strain. Likewise, these tumors are not described with oral administration of butter yellow (23) or with 10 per cent casein diet (9). These facts, together with the observed local reaction to this substance, make it seem probable that continuous local application of butter yellow for a prolonged period is responsible for the final appearance of these neoplasms.

The identity of the cell of these tumors cannot be determined with certainty from this material. The cells arise in the perifollicular envelope, which, as Kruimblaar (18) points out, contains a preponderance of large pale cells, most probably young lymphocytes, though cells of the monocytic or myeloid series cannot be excluded. The cells of these tumors seem most to resemble young lymphocytes, and the tumor thus appears to fall into the group classified by Gall and Mallory (7) as lymphoblastic lymphoma.

Kirschbaum (17) considers lymphosarcomas in the rodent to be a closely related manifestation of lymphatic leukemia, which latter disease has been extensively studied and induced in susceptible animals in a variety of ways (17). However, this close neoplastic relationship may or may not prove to be the case (5). Leukemia, probably of monocytic type, together with a variety of local neoplasms, has been induced in mice by intrasplenic injection

of carcinogenic hydrocarbons (1, 6). Likewise, methylcholanthrene has been shown to reduce the latent period in mice susceptible to spontaneous leukoses (20). However, a survey of literature (11) shows distinct lymphosarcomas of one organ to have been produced but rarely. These are discussed by Kaplan (13, 14), who induced these tumors in the thymus by x-ray and, with removal of the thymus, in the spleen later in life. The significance of the unicentric origin of this tumor in contrast to the diffuse origin of leukemic processes is discussed by Kaplan.

It might be objected that the splenic lymphoblastic reaction represents part of a local inflammatory and foreign-body reaction and is not a distinct neoplasia. However, the neoplastic property of this tissue is demonstrated, first, by invasion of the diaphragm in 1 rat, and second, by metastasis in 2 cases, one to liver and the other to liver, kidney, and lymph nodes.³

Then, having conceded the neoplastic and invasive properties of the tissue, we might still argue that this is actually a tumor of liver cells, the hepatic cell masses being primary, rather than metastases from the spleen. It might be objected that this is produced by direct action of butter yellow on hepatic cells, reaching the liver by portal flow from dietary source or from the spleen, where it is taken up by macrophages and so reaches the splenic vein. However, this concept is contradicted by several points. First, a distinct splenic mass was present, grossly in 1 case and microscopically in 4. In 1 this tumor invaded the diaphragm above the spleen. The mass in all 5 cases was composed of a uniform cell type: a young lymphocyte. Second, the tumor tissue in the liver consisted of this same young lymphocyte, which is not a type of cell produced by hepatic or bile duct neoplasia but is consistent with splenic origin. Third, these tumor nodules were widespread within the liver, in

³ Two of the animals of group 3, that is, with splenic neoplasia without metastasis, received no butter yellow by mouth. The remaining animal of this group and the 2 animals of group 4, that is, showing splenic neoplasms with metastases, received butter yellow by mouth as well as by local implant.

FIG. 6.—Changes called group 4: a lymphosarcoma of the spleen. This was found at 446 days and here shows a few groups of mature lymphocytes, remnants of Malpighian corpuscles. This spleen was not grossly enlarged. Metastases of the tumor occurred in the liver. $\times 320$. H and E stain.

FIG. 7.—Cell detail of the tumor shown in Fig. 6. These are large cells with abundant cytoplasm, free of granules (with Giemsa stain) and of inclusions. No reticulum is formed by these cells. Nuclei are round or folded with prominent, large nucleoli. Mitoses are seen fairly often. $\times 860$. H and E stain.

FIG. 8.—A nodule in the liver from the splenic tumor shown in Fig. 6. $\times 160$. H and E stain.

FIG. 9.—A small part of the splenic tumor, group 4, a lymphoblastic lymphosarcoma, found at 461 days. This spleen weighed 27 gm. Metastases were widespread. Cell structure is similar to that of Fig. 7. $\times 860$. H and E stain.

FIG. 10.—A portion of the tumor in the kidney. $\times 320$. H and E stain.

FIG. 11.—A portion of the liver in the animal shown in Figs. 9 and 10. This shows infiltration of the portal areas and, in addition, widespread distribution throughout the liver parenchyma. The cells are identical with those of the splenic mass, that is, young lymphocytes. $\times 160$. H and E stain.

distribution resembling a metastatic rather than a primary tumor. Fourth, the same tumor tissue involved lymph nodes generally in 1 case and the kidney, an organ in which it was obviously metastatic. Fifth, supporting evidence is drawn from the similar appearance and similar metastatic distribution of those lymphosarcomas in mice described by Kaplan (14) and produced by quite different experimental procedure.

SUMMARY

Five neoplasms of the spleen, classified as lymphoblastic lymphosarcomas, occurred among 28 rats bearing intrasplenic pellets of *p*-dimethylaminoazobenzene, none occurring in the controls. Two of these tumors had metastasized at the time that the animals were sacrificed, the last after 461 days. The local tissue reaction and the cells involved in the consequent neoplastic growth are described, and the significance of this process in the nature of lymphosarcoma is discussed.

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New Books

Cold Spring Harbor Symposia on Quantitative Biology, Vol. XII. Cold Spring Harbor, L.I., New York: Biological Laboratories, 1947.

The Biological Laboratory at Cold Spring Harbor continues its annual publication of its high-quality symposia on topics of biological interest with this new volume. The current volume is devoted to nucleic acids and nucleoproteins and contains some twenty-five papers by leading authorities on important aspects of this subject. The discussion is pitched for the expert in the field. The biochemistry is limited to a discussion of controversial issues in this complex subject. Most of the papers concern themselves with the relation of various forms of nucleic acids to the function of the cell. Considerable space is given to the interrelation of nucleoproteins and nucleic acids to the properties of bacteria, viruses, and phage. The volume is of timely interest to those who wish to pursue the manifold ramifications of this difficult subject in all its fine branches.

Advances in Enzymology and Related Subjects of Biochemistry, Vol. VIII. Edited by F. F. Nord. New York: Interscience Publishers, Inc., 1948.

The eighth volume of *Advances* continues at its usual high level. The chapter on the functioning of the cytoplasm by Monne discusses the detailed structure of cytoplasm, including chromidia, cytoplasmic fibrils, mitochondria, and Golgi bodies in relation to such functions as catabolic activity, contractility, motility, and permeability. Heidelberger presents a comprehensive article on the quantitative studies of complement, discussing the rigorous application of microanalytical methods to the study of the baffling complement complex. Chaikoff and Enteman give a review of the important and interesting subject of the present status of the antifatty-liver factor of the pancreas, including a discussion of its preparation and mechanism of action. Of interest to the pharmacologist is the study of Dawson on alkaloid biogenesis. The cycle which destroys the most abundant organic compound in nature—cellulose—by microorganisms is presented at length by Nord and Vitucci. Three sections are devoted to the chemistry of fats. One by Kleinzeller on the synthesis of lipids—a subject of increasing interest now that the intermediary metabolism of fatty acid is beginning to be better understood. A second section is by Breusch on the biochemistry of fatty acid catabolism. Emphasis is upon the recent *in vitro* studies of fatty acid intermediary metabolism, with special discussion of the catabolism of the two-carbon fragment resulting from the oxidation of fatty acids. There is also included an attempt to formulate a comprehensive theory of the known mechanisms of enzyme action on fatty acids involving

sterometric concepts. The recent renewal of interest in lipoxidase and auto-oxidation of fatty acids is furthered by a third section by Bergstrom and Holman devoted to this subject. The concluding chapter is by Zeller and is devoted to enzymes of snake venoms and their biological significance. Here is included discussions of esterases, hyaluronidase, and proinvasin—all topics of timely interest.

C. O. Jensen, Selected Papers. First Volume, 1886–1908. Edited by M. CHRISTIANSEN and H. O. SCHMIT-JENSEN. Copenhagen: Einar Munksgaard, 1948. Pp. 681+14 illus.

Carl Oluf Jensen (1864–1934) is best known to English-speaking readers for his pioneer work in experimental cancer, accomplished around the turn of the century. In this first of two volumes of selected studies, six of the thirty-seven articles are on cancer, but there are also six on tuberculosis and eight on the typhoid-colon group. These two groups and the remaining seventeen articles on various bacteriological subjects reflect Jensen's enduring activity at the Royal Veterinary and Agricultural College. Of the thirty-seven articles selected, twenty-four are in German; the other thirteen (including two of the cancer group) have been translated from the original Danish into English for publication in this book.

"Selected papers" of eminent scientists, as a rule, are useful tools. Especially is this so in the case of translations of Scandinavian and Slavic articles, so that this volume should be a welcome addition to a cancer library.

Fundamentals of Immunology. By WILLIAM C. BOYD. 2d ed. New York: Interscience Publishers, Inc., 1947. Pp. 503+50 illus. \$6.00.

The second edition of this universally accepted standard introductory text in immunology has retained in their entirety all those outstanding features of presentation and organization that characterized the original work. The two most notable improvements in its organization are the addition of an excellent summary at the end of each chapter and the elimination of passages in small type, with retention of all the essential subject matter originally contained in these. The bibliographies at the ends of chapters have been brought more nearly up to date and continue to provide a very broad coverage of fundamental source material.

As is to be expected in a revision of a work on a subject of this sort, where most of the elementary basic principles were elucidated prior to publication of the first edition, not a great deal of new material has been

added, though much of the old material has been worked over and re-written in the light of more recent experimental data. One important addition of new material is a thoroughgoing discussion of the Rh antigen and all its medical implications. Mention is also made of such recent advances as the methanol precipitation method of purifying bacterial toxins and the use of ultraviolet irradiation for inactivation of various virus vaccine preparations.

This book can be highly recommended as collateral reading for all graduate students in the medical sciences, medical and veterinary students; and, while it was written primarily as an introduction to the subject of immunology, its wealth of basic material and its comprehensive bibliographies make it a valuable reference work for the more advanced student of immunology as well.

Submicroscopic Morphology of Protoplasm and Its Derivatives. A. Frey-Wyssling. Translated by DR. J. J. HERMANS (Gronigen, Netherlands) and MISS M. HOLLANDER (Selborne, England). New York, Amsterdam, London, and Brussels: Elsevier Pub. Co., Inc., 1948. Pp. 236+161 illus. \$6.00.

This represents a new edition of the author's *Submicroscopic Morphologie des Protoplasmas und seiner Derivate*, published in 1938. There has been a complete revision, bringing the material up to date with advances in this field. The work is presented in three parts. The first section surveys such methods as x-ray patterns, anisotropy of swelling, polarization microscopy, and electron microscopy. The second section summarizes our present knowledge of the submicroscopic structure of cytoplasm, nucleoplasts, and erythrocytes. The third section discusses specialized derivatives of protoplasm, such as cell walls, silk, horn, sinews, muscle, and nerve.

In the author's Foreword he emphasizes that the "book is written not for specialists, but for students who are attracted towards this interesting field of research."

This is a well-written and nicely organized monograph that does not intend to be exhaustive. Rather, it is stimulating through the problems presented and left unanswered. The field of electron microscopy is adequately covered but is not presented as a complete review of the method or of the results obtained with it. The author stresses that the older, more indirect methods must be treated as equivalent means of research and that the polarizing microscope and x-ray camera are more accessible to the average biologist than is the electron microscope.

This volume is a valuable addition to the literature on the submicroscopic morphology of protoplasm, a field that is rapidly progressing and one that holds tremendous promise.

Bilharzial Cancer: Radiological Diagnosis and Treatment. By MAHMOUD AHMED AFIFI, M.B., Ch.B. (Cairo), M.R.C.S. (England), L.R.C.P. (London), D.M.R.E. (Cambridge); former director of the Radiological and Electro-therapeutic Departments of the Egyptian Government Hospital, Alexandria. London: H. K. Lewis & Co., Ltd., 1948. Pp. 111+60 illus. Cloth.

This book deals with the possible carcinogenic role of bilharziasis and the diagnosis and treatment of bilharzial cancers. It is of interest to clinicians, especially radiologists, urologists, and surgeons. All persons thinking about the causation of cancer should find the first part extremely interesting. In Egypt many rural dwellers are infected with blood flukes (*Schistosoma*), and some exhibit clinical bilharziasis. In some of the organs so infected, cancer is common, and many of the cancers show ova of the parasite. Such evidence as exists for an etiologic relationship between bilharziasis and cancer is statistical and inconclusive. The evidence is best for bladder and rectal cancer. Some organs which show much bilharziasis have little cancer in Egypt. Reviewers rarely suggest that a book should be enlarged; but this monograph would profit by the addition of control data on schistosomiasis in other diseases of the bladder, rectum, and colon and in other tissues. The relationship of parasite to tumor is worthy of further study.

ASSOCIATION NOTICE

The fortieth annual meeting of the American Association for Cancer Research will be held at the Fort Shelby Hotel in Detroit on Saturday and Sunday, April 16 and 17, 1949. Information concerning the meeting and cards for reserving rooms may be obtained from the secretary of the Association,

CHARLES W. HOOKER
Emory University School of Medicine
Emory University, Georgia

Diet and Azo Dye Tumors: Effect of Diet During a Period When the Dye is Not Fed*

C. C. CLAYTON,† PH.D., AND C. A. BAUMANN, PH.D.

(From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison 6, Wisconsin)

Although it is well recognized that rats fed azo dyes develop tumors at rates that depend upon the diet fed with the dye (28), the mechanism by which this is accomplished is unknown. One possibility is that pertinent diets alter the metabolism of the dye and thus alter the concentration of effective carcinogen; another, that diet can effect changes in normal cellular constituents that either enhance or minimize the sensitivity of the tissue to the carcinogen. Still another possibility is that appropriate diets might modify those phases of the carcinogenic process that continue after the carcinogen is no longer present in the tissues. Dietary effects in the latter categories would have a wider significance in the cancer problem than those in the first. Accordingly hepatic tumors were induced in rats by the feeding of a synthetic ration containing the potent azo dye, *m'*-methyl-*p*-dimethylaminoazobenzene (*m'*DAB), interrupted by the feeding of experimental diets containing no dye. In a few parallel studies the riboflavin contents of the livers and the coagulability of liver homogenates were also measured.

METHODS

Comparable groups of 12 to 15 young adult rats (usually males) weighing approximately 200 grams were placed in wire bottom cages and given food and water *ad libitum*. The consumption of food was measured at intervals on all groups. In experiments involving caloric restriction the rats

were kept in individual cages and the desired amount of food was fed daily. The basal diet was that used in previous studies (8, 12, 28): extracted casein 12, salts 4, corn oil 5, and glucose monohydrate (Cerelose) to 100 with vitamins added at the following levels in mg./kg. of diet: thiamine 3, pyridoxine 2.5, calcium pantothenate 7.5, riboflavin 2, and choline 30. In the final dietary series (Table 2, groups 29 to 33) and in the analytical series (Table 4) all diets contained 1000 mg. of choline per kg. Each rat also received 2 drops of halibut liver oil every 4 weeks.

For the incorporation of *m'*-methyl-*p*-dimethylaminoazobenzene (*m'*DAB) into the rations, the dry ingredients including the vitamins were mixed first and an ether solution of the dye was evaporated onto the dry mixture. The corn oil was then incorporated into the diet and the mixture passed through a 10-mesh sieve. The concentration of dye in the ration and the periods of time the dye was fed are described below.

EXPERIMENTAL AND RESULTS

Tumor incidence after interrupted feeding of azo dyes.—In the initial experiment *m'*-methyl-*p*-dimethylaminoazobenzene (*m'*DAB) was fed at a level of 0.048 per cent to 3 groups of rats for a total of 10 weeks. The control groups received the dye continuously. Two other groups received the dye for 6 weeks followed by "periods of interruption" of 4 or 10 weeks during which the basal diet free of the dye was fed. Thereafter the carcinogen was fed for 4 more weeks, after which the dye-free diet was fed, and the experiment was terminated at the end of 29 weeks. Thus the 3 groups received the basal diet for 19, 15, and 9 weeks respectively after the last exposure to the carcinogen.

* Published with the approval of the director of the Wisconsin Agricultural Experiment Station. Supported in part by the Jonathan Bowman Cancer Fund and by a grant from the Committee on Growth, American Cancer Society.

† United States Public Health Service Predoctorate Research Fellow.

Liver tumors developed in all groups, although the interruption of the carcinogenic treatment reduced tumor incidence somewhat. In the group receiving the dye continuously, 85 per cent of the rats developed tumors; when the feeding of the dye was interrupted for 4 or 10 weeks, the incidences of tumors were 58 per cent and 61 per cent respectively (Table 1, groups 1 to 3). The results were similar in 2 other series in which 0.064 per cent of *m'*-DAB was fed for 8 weeks with "periods of interruption" ranging from 2 to 12 weeks after an initial exposure to the dye of 4 weeks. Groups receiving the carcinogen for only 4 weeks failed to develop tumors (Table 1, groups 4 and 8). The incidences of tumors were 87 and 100 per cent in the groups receiving the dye continuously (Table 1,

High casein and riboflavin.—The levels fed during the period of interruption were 24 per cent of casein and 20 mg. of riboflavin per kg. of diet. In the first series there was a definite decrease in tumor incidence in the group fed the more adequate diet: hepatic tumors developed in 47 per cent of the group receiving the basal diet during the intermediate period as compared to 20 per cent of the animals receiving supplements of casein and riboflavin (Table 2, groups 14 and 18). A second series indicated a smaller difference between the percentages of tumor incidences, 90 vs. 77 per cent (Table 2, groups 23 and 24). In this series the survival of the rats in the control group was poor and the number of animals that developed tumors in the group receiving the added casein and riboflavin

TABLE 1

LIVER TUMORS PRODUCED AFTER INTERRUPTED FEEDING OF *m'*METHYL *p*-DIMETHYLAminoAZOBENZENE
Groups 1-3 0.048% *m'*DAB 6 wks.; Basal Diet x wks.; 0.048% *m'*DAB 4 wks.; Basal to 29 wks.
Groups 4-13 0.064% *m'*DAB 4 wks.; Basal Diet x wks.; 0.064% *m'*DAB 4 wks.; Basal to 29 wks.

Group	Weeks on basal (x wks.)	Av. init. wt. gm.	Av. wt. end 1st dye feeding gm.	Av. wt. start 2d dye feeding gm.	Av. wt. end of dye feeding gm.	Av. food consump- tion on dye feeding gm./day	Survival at end of dye feeding	No. of tumors 29 wks.	Neg. survivors 29 wks.	Cirrhosis at 29 wks.	Per cent Tumors
1	0	160			183	9.8	13/15	11	1	0	85
2	4	157	182	243	220	10.2	12/15	7	4	0	58
3	10	155	184	249	236	9.3	13/15	8	3	0	61
4*	0	157	139			7.7	15/16	0	12	0	0
5	0	177			167	9.0	15/17	13	1	0	87
6	4	170	150	214	197	9.2	15/17	7	4	4	47
7	8	172	155	232	212	9.3	15/18	5	4	6	33
8*	0	194	176			6/6	0	5	1	0	0
9	0	202			197	11/12	11	0	0	0	100
10	2	184	162	209	198	11/15	9	2	0	0	82
11	4	181	162	231	221	10/15	9	0	1	1	90
12	8	191	168	272	247	13/15	7	5	0	0	54
13	12	189	171	300	276	9/15	6	1	2	0	67

* Groups 4 and 8 received the dye for only 4 weeks.

groups 5 and 9), while in groups in which the administration of the carcinogen was interrupted, the percentages of tumors ranged from 33 to 90 per cent (Table 1, groups 6, 7 and 10 to 13). In general the number of tumors decreased as the period of interruption was extended. However, when the period of interruption was 4 or 8 weeks, tumor incidences were sufficiently high to warrant the use of this procedure in nutritional studies.

EFFECT OF DIET DURING THE PERIOD OF INTERRUPTION

In a typical experiment *m'*DAB was fed at a level of 0.064 per cent for 4 weeks followed by 4 weeks during which various dye-free diets were fed; the basal diet containing the *m'*DAB was then fed for 4 more weeks after which the dye-free basal diet was fed for 8 weeks when the animals were killed for examination. The group fed the basal diet during the period of interruption served as a control.

was actually greater than that in the control group. In a third series the incidence of tumors was essentially the same in the group fed extra casein and riboflavin during the period of interruption as in the control group (62 vs. 69 per cent; Table 2, groups 29 and 32). Thus it is evident that riboflavin was much less anticarcinogenic in the present experiments than in previous studies in which it was fed simultaneously with *p*-dimethylaminoazobenzene (15, 23), *p*-monomethylaminoazobenzene (20), *o'*-methyl-*p*-dimethylaminoazobenzene (8), or *m'*-methyl-*p*-dimethylaminoazobenzene (8). It is possible, therefore, that much of the effect of the vitamin is concerned with the detoxification of the azo dye or with resisting its immediate effects.

Effect of choline, methionine, and nicotinamide.—Group 26, Table 2, was fed 0.1 per cent of choline during the period of interruption. This is not a particularly high level of the vitamin in terms of current nutritional practice, but it is significantly

higher than the 0.003 per cent in the control diet. To two other groups 0.72 per cent of methionine was fed in the diet; this is the amount of methionine in a diet containing 24 per cent of casein (33). Still other groups received 0.29 per cent of nicotinamide added to the basal diet from which all choline was removed; this amount of nicotinamide could theoretically combine with all of the methyl groups in the 12 per cent of casein in the diet.

but the number of rats with tumors was actually greater in the methionine fed group than in the controls, due to the relatively poor survival of the latter (Table 2, groups 25 and 27). Thus attempts to minimize cirrhosis during the period of interruption by feeding choline and methionine had little effect on the final incidence of liver tumors, possibly because the degree of recovery after 4 weeks was already maximal in the control animals.

TABLE 2

LIVER TUMORS DUE TO m'DAB: EFFECT OF DIET DURING THE PERIOD OF INTERRUPTION
 Groups 14-18, 23-33 0.064% m'DAB 4 wks.; Dye-free diets 4 wks.; 0.064% m'DAB 4 wks.; basal 8 wks.
 Groups 19-22 0.064% m'DAB 4 wks.; Dye-free diets 4 wks.; 0.018% m'DAB 4 wks.; basal 8 wks.

Group	Diet during period of interruption	Av. wts.		Food intake on dye-		Av. wts.		Food intake on dye-		Survival at 12 wks.	Tumors at 20 wks.	Survivors at 20 wks.	Cure%	Per cent tumors
		0 wks.	4 wks.	free diet	gm./rat/ day	8 wks.	12 wks.	free diet	gm./rat/ day					
14	Basal	170	150	13.6	214	197	9.2	15/17	7	4	4	4	47	
15	0.29% nico- tinamide	166	150	12.3	188	177	8.2	14/18	9	1	3	0	64	
16*	"	167	146					8.2	17/17	0	11	0	0	
17	0.72% methio- nine	163	145	11.4	208	198	9.7	15/15	8	5	2	2	53	
18	24% casein 20 mg. ribofl./kg.	161	146	12.1	223	205	8.9	15/15	3	8	2	2	20	
19	Basal	228	186	13.9	245	229	9.9	10/15	4	2	2	2	40	
20	Basal restr. (63%)	220	171	8.8	183	210	10.2	9/15	6	1	2	2	67	
21	0.29% nicotina- mide	228	181	11.3	222	234	11.8	11/15	10	0	1	1	91	
22	5 ppm. selenium	223	160	10.8	198	214	11.6	9/15	2	3	3	3	22	
23	Basal	181	162		231	221		10/15	9	0	1	1	90	
24	24% casein 20 mg. ribofl./kg.	198	171		276	247		13/15	10	3	0	0	77	
25	Basal	193	165	11.7	211	198	10.3	9/14	7	2	2	2	78	
26	0.1% Choline	209	176	15.7	226	218	10.9	12/15	9	3	3	3	75	
27	0.72% methio- nine	204	169	15.8	231	209		12/15	8	4			67	
28	0.29% nicotina- mide	201	175	13.0	220	208		9/15	6	2			67	
29	Basal	193	189	14.5	256	244	12.2	13/15	8	4	1	1	62	
30	Basal restr. (63%)	197	178	9.1	192	207	10.7	14/15	13	0	1	1	93	
31	5 ppm. selenium	201	184	11.3	226	230	12.0	13/15	4	6	3	3	31	
32	24% casein 20 mg. ribofl./kg.	200	200	15.7	308	279	13.7	13/15	9	3	1	1	69	
33	20% corn oil	194	193	14.3	256	242	13.0	15/15	15	0	0	0	100	

* Group 16 received dye for only 4 weeks.

The increased level of choline in the diet during the intermediate period had no effect on the final tumor incidence; tumors developed in 75 per cent of the animals receiving the higher level of choline as compared to 78 per cent incidence in the group receiving the basal diet (Table 2, groups 25 and 26). Methionine likewise appeared to be without effect on the final tumor incidence when fed during the period of interruption. In the first series the incidence of tumors was 47 per cent in the control group as compared to 53 per cent when methionine was fed (Table 2, groups 14 and 17). In a second series the percentage of tumors in the group receiving methionine was less than in that receiving the basal diet during the period of interruption,

On the other hand the incidence of tumors appeared to be increased when the diet fed during the intermediate period was made low in available methyl groups by the addition of nicotinamide. In one series the incidence was increased from 47 per cent in the group receiving the basal diet to 64 per cent in the group that had received nicotinamide (Table 2, groups 14 and 15). In another series 91 per cent of the animals on the low methyl diet developed tumors as compared to only 40 per cent of the control animals (Table 2, groups 19 and 21). In a third series the survival of the animals was poor, and only 8 of 15 animals in the nicotinamide supplemented group were alive at the termination of the experiment. In this series no increased num-

ber of tumors was seen in the group fed nicotinamide (Table 2, groups 25 and 28). The results of the three series, however, suggest that a dietary regime which prolongs eirrhosis during the period of interruption, thereby tends to increase the final incidence of hepatic tumors due to *m'DAB*. It is of interest in this connection that neither choline (20, 39), methionine (12, 39), nor nicotinamide (20) affected the incidence of liver tumors when these substances were fed with the carcinogen *p*-dimethylaminoazobenzene or *p*-monomethyl-aminoazobenzene.

Effect of caloric restriction.—Of all the dietary factors that have been studied in connection with carcinogenesis, caloric intake seems to have the widest effect; a reduced caloric intake decreased the rate of formation of spontaneous tumors of the lung (35) or mammary gland (36, 38), of skin tumors induced by hydrocarbons (36) or ultraviolet light (30), of subcutaneous tumors due to hydrocarbons (35, 36) and of leukemia (34) in susceptible strains of mice. Preliminary attempts have been made (7) to alter the caloric intake of rats receiving *p*-dimethylaminoazobenzene (DAB) and at the same time to insure an approximately equal intake of the carcinogen by the restricted groups and those fed *ad libitum*, since the carcinogenicity of the azo dye varies with the level of intake. Unfortunately, however, an equivalent intake of dye by the groups restricted in calories could only be achieved by increasing the percentage of azo dye in the ration, and animals fed such concentrations failed to survive.

The records of the voluntary intake of food by rats receiving azo dyes in protective or non-protective diets (Table 3) fail to reveal any constant relationship between caloric intake and carcinogenicity. When the vitamin B₆ content of the ration was varied, a decreased consumption of food (and azo dye) was associated with a decreased tumor incidence (21). An increase in the percentage of corn oil from 5 to 20 per cent resulted in a decreased caloric intake, a decreased intake of dye, but nevertheless a marked increase in tumor formation (16). On the other hand, rats fed hydrogenated coconut oil voluntarily consumed more food and carcinogen than the control animals but developed fewer tumors (8, 20, 22). The addition of riboflavin to diets containing azo dyes usually results in an increase in the intake of calories and of carcinogen while the incidence of tumors is decreased. In all of these experiments, however, dietary factors other than calories were varied, and the only conclusion that emerges is that the composition of the diet apparently exerts more effect on the development of hepatic tumors than the amount of food eaten.

However when the feeding of an azo dye is interrupted, it is possible to alter the caloric intake of groups of animals during the period of interruption and still give equal amounts of azo dye to the restricted and the control groups during the preliminary and subsequent periods. In the present experiment the restricted groups were given 62 to 63 per cent as much of the basal ration during the period of interruption as consumed by the groups fed *ad libitum*. The restriction therefore was in all nutrients present in the basal ration rather than in calories alone. Tannenbaum (37) and Rusch *et al.* (32) have shown that for other tumors the effects of restriction are essentially the same whether decreased amounts of a complete ration are fed or whether the restriction is solely in the fat and carbohydrate in the diet with an automatic increase in the percentage of all other ingredients. In the present study some restriction in food intake was also necessary after the feeding of the azo dye was resumed. The control groups were well nourished during the period of interruption, and voluntarily ate less when the feeding of azo dye was resumed. On the other hand, rats that had been partially starved during the period of interruption tended to "make up" for lost calories when given unlimited amounts of diet containing the azo dye. It was therefore necessary to equalize the intake of food and carcinogen during the first 10 to 14 days of the second dye feeding period, after which the consumption of food by the two groups was very similar.

The rats restricted in calories during the period of interruption developed significantly more tumors than the unrestricted rats of the control group. In the first series the percentage of tumors was increased from 40 in the control group to 67 in that restricted in calories during the intermediate period (Table 2, groups 19 and 20); in the second series (groups 29 and 30) the incidence was raised from 62 per cent in the control group to 93 per cent in the restricted group. Thus, in contrast to the general observation that caloric restriction decreases the development of other types of tumors caloric restriction during the intermediate period increased the development of hepatic tumors in the present experiment.

Effect of selenium.—In view of the reported carcinogenic activity of selenium (25), its ability to damage the liver (19, 24), and a possible additive effect between selenium and the azo dyes (26), 5 parts per million of selenium as sodium selenite were fed in the present experiment during a 4 week period of interruption between two 4 week periods during which 0.064 per cent *m'DAB* was fed. In two separate experiments there was a reduction of about 50 per cent in the incidence of liver tumors

when selenium was fed during the intermediate period. The incidences of tumors in the groups fed selenium and in the control groups were 22 vs. 40 per cent in one series and 31 vs. 62 per cent in the second (Table 2, groups 22 and 19, 31 and 29). The animals receiving the selenium did not gain as much weight during the intermediate period as the animals on the basal diet and during the second dye feeding period they actually gained weight as compared to a loss of weight by the animals that

Effect of 20 per cent of corn oil.—In a previous experiment tumor formation was hastened by a diet containing 20 per cent of corn oil fed simultaneously with DAB (16). In the present experiment 20 per cent of corn oil was fed during the period of interruption between two 4 week periods during which *m'DAB* was fed in the regular basal diet containing 5 per cent of the oil. All of the rats fed the higher level of corn oil developed tumors as compared with an incidence of only 62 per cent in

TABLE 3
VOLUNTARY VARIATIONS IN CALORIC INTAKE BY RATS INGESTING AZO DYES
IN PROTECTIVE AND NON-PROTECTIVE DIETS

Azo dye	Diet	Avg. food consumption cm. rat day	Avg. daily caloric intake	Per cent tumors	Reference
DAB	2.5 mg. kg. B ₁₂	11.8	33.1	92	21
-	0.2 "	7.6	29.0	7	
-	Control	6.5	24.8	67	23
-	Vitab	7.7	29.0	100	
-	" + riboflavin	8.7	32.8	0	
-	Control	10.0	38.1	50	11
-	" low riboflavin	7.5	28.6	55	
-	Control	10.1	38.1	73	22
-	HCNO	11.3	33.1	7	
-	Low fat	11.0	39.1	5*	16
-	5% corn oil	9.7	37.0	73*	
-	26% corn oil	7.3	33.7	100*	
-	20% lard	7.8	36.0	60*	
MAB	Control	11.5	33.9	87	20
-	HCNO	13.3	30.3	31	
-	0.83% Nicotinamide	9.6	30.6	89	
<i>m'DAB</i>	Control	11.7	34.6	80	8
-	Vitab	11.0	31.7	92	
-	HCNO	12.3	37.3	53	
-	High riboflavin	11.1	42.3	47	
<i>o'DAB</i>	Control	7.3	27.8	63	8
-	Vitab	7.7	29.1	73	
-	HCNO	9.3	33.3	46	
-	High riboflavin	10.1	38.5	38	

DAB = *p*-dimethylaminobenzene

MAB = *m*-nitrobenzene

m'DAB = *m*-methyl *p*-dimethylaminobenzene

o'DAB = *o*-methyl *p*-dimethylaminobenzene

HCNO = Hydrogenated coconut oil

Vitab = Basal diet concentrate

* Tumor incidence after 4 months of dye feed (2).

had received the basal diet during the intermediate period. The slower gains by the animals receiving the selenium during the intermediate period were due in part to a decrease in food intake. Subsequently when selenium was replaced by the azo dye, the consumption of food and dye by the rats previously exposed to selenium was either equal to or greater than that by the control groups. Hence the reduced tumor incidence could not be ascribed to a diminished intake of carcinogen. Furthermore the decreased intake of food during the intermediate period would have tended to increase the ultimate incidence of tumors (Table 2, groups 20 and 30). Thus the inhibiting effect of selenium on tumor developments was evident in spite of accompanying influences in the opposite direction.

the control group (Table 2, groups 29 and 33). Thus the high level of corn oil was approximately as effective in stimulating tumor formation when fed in the absence of the dye as in previous experiments in which a dye and the oil were fed simultaneously.

*Hepatic riboflavin and coagulability as affected by diet during the period of interruption.*³—As one approach to the means by which the diet fed during the period of interruption affects tumor incidence, fluorometric determinations (1, 5) were made of riboflavin in the livers of rats fed 0.064 per cent of *m'DAB* for 4 weeks followed by 4 weeks on the various diets under consideration. Livers from

³ W. L. Miller, Jr., assisted in some of the analytical experiments.

rats fed high amounts of casein and riboflavin contained the highest concentrations of the vitamin, 32.4 γ /gram (Table 4). Rats fed a decreased amount of food likewise contained a high concentration of riboflavin, 29.9 γ /gram of liver, although the total amount of vitamin per liver was low due to the small size of this organ. This confirms previous observations (9). The concentra-

tions of hepatic riboflavin in the groups fed selenium or 20 per cent of corn oil during the period of recovery were essentially the same as those in the control group, 22.7 γ /gram, while rats fed nicotinamide contained relatively low concentrations of the vitamin in the liver, 17.9 γ /gram (Table 4). Feeding of the basal diet for 8 weeks after the dye feeding period only increased the concentration of riboflavin slightly (24.5 γ /gram) over that found at 4 weeks (22.7 γ /gram), and 12 weeks on the basal diet had no further effect (23.5 γ /gram).

TABLE 4
HEPATIC RIBOFLAVIN AND HEAT COAGULABILITY OF LIVER HOMOGENATES FROM RATS FED
m'DAB FOLLOWED FOR VARIOUS PERIODS BY DIETS FREE FROM DYE

DIET AND TIME	INIT. WT. gm.	FINAL WT. gm.	FOOD INTAKE on dye gm./day	FOOD INTAKE dye-free gm./day	LIVER	RIBOFLAVIN γ /liver	RIBOFLAVIN γ /gm.	COAG. MINUTE
0.064% <i>m'DAB</i> 4 wks.	235	180			Normal	91	16.5	
	202	201			"	88	16.5	
	295	245			"	128	15.9	
0.064% <i>m'DAB</i> 8 wks.	264	209	9.9		Normal	102	16.3	
	247	201			Cirrh.	125	16.2	35+
	202	241			"	146	12.9	35+
0.064% <i>m'DAB</i> 4 wks. Basal 2 wks.	305	246			"	119	12.5	35+
	271	229	8.7		Normal	130	13.9	35+
	250	255			"	158	18.4	
0.064% <i>m'DAB</i> 4 wks. Basal 4 wks.	253	263			"	167	18.9	
	325	334			"	196	21.6	
	276	264	10.6	15.0	Normal	174	19.6	
0.064% <i>m'DAB</i> 4 wks. High casein and ribo- flavin 4 wks.	235	274			"	216	22.7	35+
	251	272			"	200	21.6	35+
	308	300			"	220	23.9	35+
0.064% <i>m'DAB</i> 4 wks. Nicotinamide 4 wks.	265	282	9.2	15.5	Normal	212	22.7	35+
	295	334			"	329	26.1	7
	263	280			"	425	39.3	10
0.064% <i>m'DAB</i> 4 wks. Selenium 4 wks.	232	292			"	344	31.8	4
	263	302	9.1	16.7	Fatty	366	32.4	7
	245	259			"	236	19.7	30+
0.064% <i>m'DAB</i> 4 wks. Selenium 4 wks.	252	265			"	233	17.5	30+
	301	290			"	238	16.6	30+
	266	271	10.6	17.4	Normal	236	17.9	30+
0.064% <i>m'DAB</i> 4 wks. Basal restr. 4 wks.	243	209			"	173	18.8	30+
	263	212			"	163	21.4	8
	288	280			"	230	21.3	30+
0.064% <i>m'DAB</i> 4 wks. 20% corn oil 4 wks.	265	234	8.0	11.3	Normal	189	20.5	8-30+
	239	197			"	142	29.6	3
	271	216			"	137	29.2	25
0.064% <i>m'DAB</i> 4 wks. 20% corn oil 4 wks.	291	219			"	166	30.8	11
	267	211	9.9	9.0	Normal	148	29.9	13
	227	259			"	195	22.2	30+
Av.	254	289			"	208	22.9	30+
	213	308			"	210	22.1	30+
	231	285	10.0	14.4		204	22.4	30+

m'DAB = *m'* methyl *p*-dimethylaminobenzene.

tions of hepatic riboflavin in the groups fed selenium or 20 per cent of corn oil during the period of recovery were essentially the same as those in the control group, 22.7 γ /gram, while rats fed nicotinamide contained relatively low concentrations of the vitamin in the liver, 17.9 γ /gram (Table 4). Feeding of the basal diet for 8 weeks after the dye feeding period only increased the concentration of riboflavin slightly (24.5 γ /gram) over that found at 4 weeks (22.7 γ /gram), and 12 weeks on the basal diet had no further effect (23.5 γ /gram).

Thus the concentration of hepatic riboflavin at the end of the period of interruption did not appear to be the primary factor affecting theulti-

restricted in calories a high concentration of riboflavin was associated with an increased incidence of tumors.

Parallel studies were made of the coagulability of homogenates from the same livers analyzed for riboflavin. Tumor homogenates or liver homogenates from rats receiving *m'DAB* for 3 or more weeks fail to coagulate upon heating, while homogenates from rats on control diets or on diets containing non-carcinogenic azo dyes coagulate in 3 to 5 minutes (10). In the present study coagulability was determined by the original method (10) except that the livers were not perfused. Liver homogenates from rats fed *m'DAB* for 4 or more

weeks failed to coagulate on heating and this failure in coagulability was evident even after a recovery period of 4 weeks on most of the diets free from dye. Homogenates from rats fed restricted amounts of the basal ration or from those fed the diet high in casein and riboflavin coagulated in 13 and 7 minutes respectively. Since coagulation is influenced by a number of factors including the total concentration of coagulable protein (10), of fatty acids (3), or of nucleoprotein (4), the relatively rapid coagulation of livers from the restricted groups is presumably due to an altered concentration of one or more of these factors.

DISCUSSION

The intermittent application of carcinogenic hydrocarbons (31, 18), of ultraviolet light (31) or of these carcinogens and appropriate co-carcinogens (2) has previously been observed to result in a reasonably high incidence of tumors. In the present study the feeding of *m'*methyl-*p*-dimethylaminonazobenzene was interrupted for periods of 1 to 3 months and a high incidence of hepatic tumors also observed. These different types of tumors therefore are similar in that the initial application of a subcarcinogenic dose produces changes which persist for a long time, with subsequent subcarcinogenic treatments being sufficiently additive to give rise to neoplasms. Further evidence for the persistence of subcarcinogenic changes in the liver of rats fed the azo dye for 4 weeks is the fact that the failure of homogenates from such livers to coagulate on heating persisted for at least 4 weeks after the administration of dye was discontinued.

The various incidences of tumors in the groups fed different diets during the period of interruption suggest that the effects of diet previously observed need not all be ascribed to an altered metabolism of the carcinogen. The increased percentage of tumors observed when the caloric intake of the rats was restricted during the intermediate period may be related to the phenomenon of liver regeneration rather than to the caloric effect *per se* that is so important for other types of neoplasms. The livers of rats restricted in calories during the period of interruption averaged only 5.0 grams in weight as compared to 9.3 grams for the controls fed *ad libitum* during this period. During the final period of dye feeding the undersized livers increased in size, and this increase was taking place while carcinogen was present in the liver. The experiment therefore is somewhat analogous to that in which proliferation in skin exposed to hydrocarbon (17) or to a virus (27) hastens carcinogenesis.

Other nutritional effects appear to be related to the condition of the liver when the second feeding of dye was begun. Cirrhosis due to an azo dye per-

sists longer when nicotinamide is fed than on an ordinary diet (20), and the rats fed nicotinamide during the intermediate period still had cirrhotic livers when the feeding of the carcinogen was resumed. Livers of rats fed 20 per cent of corn oil during the intermediate period did not appear cirrhotic although they were somewhat fatty when the feeding of the dye was resumed.

The other cirrhosis-producing agent studied, selenim, retarded the formation of tumors due to *m'DAB* in spite of the reported carcinogenicity of the element itself. However carcinogens are not always additive; examples of carcinogens that do not reinforce one another include hydrocarbons and ultraviolet light (29), nrethane and methylcholanthrene (13), and nrethane and *p*-dimethylaminoazobenzene (14). In a previous study in this laboratory no consistent additive effects between selenim and the azo dyes were observed when selenim was fed with the dye or after it (26). Attempts to demonstrate an additive effect between azo dyes and 2-necetyl-aminofluorene have likewise been unsuccessful (6).

SUMMARY

1. *m'*Methyl *p*-dimethylaminonazobenzene was fed to rats for 8 weeks, either continuously or interrupted for periods of 2 to 12 weeks. Tumors developed in the livers of all groups, the number depending on the length of the period of interruption.

2. When 0.064 per cent of the dye was fed for two 4 week periods separated by a 4 week period during which various dye-free diets were fed, the final incidence of tumors depended upon the diet fed during the intermediate period. Tumor incidence was increased by diets containing 0.29 per cent of nicotinamide or 20 per cent of corn oil or when the amount of basal diet fed during this period was restricted to 63 per cent of the calories consumed by the control group. Tumor incidence was decreased by a diet containing 5 parts per million of selenium. A diet containing 24 per cent of casein and 20 mg. of riboflavin per kg. during the period of interruption reduced the incidence of tumors in one experiment and had no effect in two others. Choline or methionine fed during the intermediate period had no effect on tumor incidence.

3. Homogenates of livers from rats fed the dye for 4 weeks failed to coagulate with heat. This decreased coagulability persisted for at least 4 weeks on most of the dye-free diets fed. The percentage of tumors that developed on the various diets did not appear to be related to the coagulability of the liver homogenates when the feeding of azo dye was resumed nor to the concentration of hepatic riboflavin at this time.

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Early Changes in the Lungs of Rats Treated with Urethane (Ethyl Carbamate)

A. ROSIN, M.D.*

(From the Department of Experimental Pathology, The Hebrew University, Jerusalem, Israel)

In the course of studies on the early changes in the livers of rats injected daily for several days with urethane (2), we noted proliferative changes in the epithelial elements of the lung. We decided to investigate the nature of these changes. In the meantime Nettleship, Henshaw, and Meyer (7) published observations on C3H mice treated for several months with weekly doses of urethane. They found a much higher incidence of lung tumors than in the untreated C3H mice and a much earlier appearance of the tumors than usual. The tumors, which were seen 2 to 3 months after the beginning of the treatment, were described as adenomata.

Jaffé (5) observed the same type of lung tumors as reported by the former authors in rats fed or injected with urethane after a period of more than 9 months. Guyer and Claus (4) found that intraperitoneal injections of urethane induced the formation of multiple pulmonary adenomata in the majority of 91 treated rats. Smaller foci which seemed to precede the tumors were detected as early as 8 to 10 weeks after the first treatment. Orr (9, 10) treated mice with urethane and found adenomata in the lungs of 58.4 per cent of the treated animals. The earliest adenomatous changes occurred after 2½ months. Noble and Millar (8) injected white mice with a 25 per cent urethane solution in 5 per cent zinc acetate. They observed lung adenomas after 3 to 4 months. Besides these tumors they found lymphosarcoma in 2 mice and a malignant hemangiopericytoma in one. Sellie and Thackray (12) observed lung tumors in 100 per cent of CBA mice 7 months after intraperitoneal injection of urethane.

In our observations adenoma-like formations in the lungs of rats treated with daily injections of urethane were observed by microscopic examination as early as 4 to 5 days after the first injection. This rapid development was rather unusual and we refrained from publishing our results. We sought for adenomata in the lungs of the normal rats of our colony without, however, finding them. Recently Smith and Rons (13) reported having ob-

served lung adenomata in newborn rats, from a mother injected with urethane several days before parturition. The tumors were seen as early as 10 days after the first administration of urethane. The early changes in the lungs of rats treated with urethane are described in the present paper.

MATERIAL AND METHODS

Albino rats weighing from 60 to 200 gm., were used for these experiments. Urethane (ethyl carbamate) in 10 per cent aqueous solution was injected intraperitoneally in doses of 1 cc. per 100 gm. body weight. Animals which did not die as a result of treatment were killed with ether. The lungs were removed, injected through the bronchi with Zenker's fluid and then fixed. Paraffin-celloidin sections 5 μ in thickness were stained with hematoxylin-eosin, Giemsa's stain, and in some cases with methyl green-pyronin.

RESULTS

On gross examination the lungs of the experimental animals were often congested and edematous, but in no instance were nodules observed. Microscopic examination revealed that 6 out of 28 rats had undoubtedly adenomatous or papillomatous formations. Seventeen showed marked proliferation of the bronchial epithelium with downgrowth into the respiratory bronchioles and alveolar ducts. In some, proliferation of the living cells of the pulmonary alveoli was observed. Five animals showed no changes at all.

The animals with undoubtedly adenomatous or papillomatous formations died or were killed 4, 5, 7, and 13 days after the first injection of urethane. The 17 rats of the second group died or were killed after 2 to 20 days. The rats which showed no changes were killed after 5 and 9 days.

Histology.—In rats treated with daily injections of urethane which died after 2 to 4 injections, i.e. after 2 to 3 days from the beginning of the experiment, edema was observed in some areas of the lung. Here the alveoli were filled with homogeneous pink-staining fluid. The blood vessels were surrounded by large ring-shaped spaces containing homogeneous pink fluid in which some distended

* Research Fellow of the Hebrew University.

fibers of connective tissue could be discerned. In other places the capillaries were engorged with red blood corpuscles and here and there hemorrhages were seen in the parenchyma. Sometimes slight emphysema was seen in the peripheral areas of the lobe. The mucosa of the large bronchi was swollen and hyperplastic. In a great number of bronchi there were large folds of redundant mucosa which sometimes filled the lumen of the bronchi. The bronchial epithelium, however, remained in a single layer. The epithelial cells of the bronchi contained several nuclei lying close together and forming rows. Mitoses were rare. Sometimes a few polymorphonuclear leukocytes could be seen in the lumen of the bronchi. The lining cells of the alveoli, especially of those which lie close to bronchi, were swollen and protruded into the alveolar lumina. Often numerous cells could be seen extruding into the lumen of the alveoli.

The most remarkable tumor-like formations were seen in animals which were treated with 4, 6, or more daily injections of urethane. In one animal which died 4 days after the first injection the major part of one lobe consisted of consolidated tissue containing all types of round and spindle cells and a few polymorphonuclear leukocytes. This solid tissue contained numerous papilloma-like formations apparently originating from bronchioles (Fig. 1). They were found in the neighbourhood of blood vessels and were generally lined for short stretches by a muscularis layer. Their size, however, did not correspond to the size of the vessels, since they were very large, extending into the lung parenchyma; they were not limited by a capsule. They contained long papillomatous processes which sometimes nearly obstructed the lumen. The papillae consisted of a small fibrous stroma and high cylindrical cells, often ciliated. In places the cylindrical cells seemed to extend into the alveolar ducts. This was especially noticeable on the border between the solid and air-containing tissue. Here large air spaces could be seen lined with cylindrical epithelium which slowly transformed into cubical epithelium and eventually flattened completely (Fig. 2). The alveolar septa in the air-containing tissue were rich in cells. There was no edema.

In another animal, killed 5 days after first injection of urethane, tumor-like formations were seen at different places and in different lobes. They were tubulo-papillomatous or adenomatous structures around a lumen. Some of these formations seemed to originate from respiratory bronchioles with highly proliferated mucosa showing papillomatous processes (Fig. 3). The papillae consisted of a very thin capillary loop with supporting connective tissue covered by a tuft of high cylindrical cells. The nu-

clei of these cells were numerous and approximately equal in size, round or oval, with fine chromatin threads and one clearly visible nucleolus. They were densely packed at the bases and in the long axes of the cells. Mitoses were scanty.

These formations were sometimes bordered for short stretches by a muscularis, while at other points no boundary was visible and there was no definite capsule (Fig. 4). Sometimes the proliferating cells invaded the neighbouring tissue, so that numerous small tubular formations could be found adjacent to one large cystadenomatous structure (Fig. 5). These small tubules were lined with cubical cells. Between the tubules numerous free proliferating cells with clear round or oval nuclei and basophilic cytoplasm could be found. The cytoplasm stained deeply pink with pyronin. Mitoses in these cells were very numerous. The whole proliferative area contained numerous histiocytes, round cells, and fibroblasts. Polymorphonuclear leukocytes were scanty.

In another lobe adenomatous structures were seen. Some of them seemed to originate from bronchioles whose epithelial cells had proliferated and grown down into the alveolar ducts. Sometimes a backward growth of epithelial elements into the adjacent lung tissue could be seen. Other adenomata showed the features of alveoli invested by a continuous layer consisting of a single row of high cylindrical cells (Fig. 6). The nuclei of the cells were regular in size, round or oval, and lay in the long axis of the cell. Mitoses were not seen. There was a slight fibroblastic reaction in the surrounding tissue.

Almost all the bronchioles of the lobe showed proliferation of the mucosa. Sometimes polypous processes in the lumina of smaller bronchi were found either free or in connection with the mucosa. The respiratory bronchioles and alveolar ducts were often covered by high cubical or columnar cells.

After 7 days in addition to tubulo-papillomatous formations, an intense proliferation of the lining cells of the pulmonary alveoli could be observed. There were alveoli lined with cubical cells, some of them in mitosis. In other places the alveolar ducts and alveoli were covered by high, cylindrical, mucus-secreting cells (Fig. 7). The nuclei of these cells were numerous but mitoses were scanty. Often several such adenomatous formations were found in groups and surrounded by fibroblastic tissue (Fig. 8). The picture resembled the "mucous epithelial hyperplasia" described in man by Taft and Nickerson (14). Sometimes transition of the high cylindrical cells to more cubical cells and eventually to the proliferating alveolar cells was clearly observed. The latter were very numerous. Mitoses were present.

In animals in which the changes were not so obvious an extensive downgrowth of cubical cells into the alveolar ducts could always be noted. In some animals there was intense proliferation of the mucosa of the bronchi so that their lumen was sometimes obstructed by epithelial masses.

In animals subjected to a course of treatment of

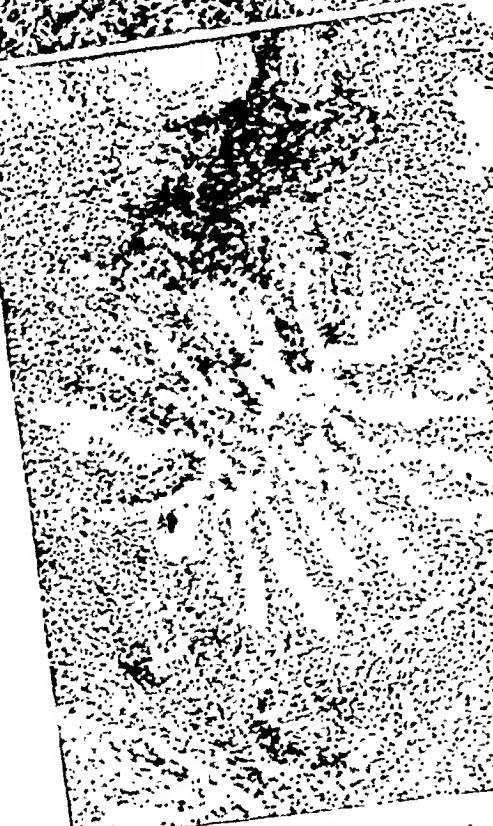


FIG. 1.—Rat 65, treated with 4 intraperitoneal injections of urethane on 4 consecutive days. The rat died 4 days after the first injection. Consolidated lung tissue containing papilloma-like formations originating from bronchioles. $\times 125$.

FIG. 2.—Same rat. Border between solid and air-containing tissue. Air spaces lined with cylindrical and cubical epithelium. $\times 500$.

All photomicrographs were made from sections stained with hematoxylin-eosin

FIG. 3.—Rat 129, treated with 5 intraperitoneal injections of urethane on 5 consecutive days. The animal was killed on the fifth day. Papillomatous structure bordered for short stretches by a muscularis layer. $\times 100$.

FIG. 4.—Same rat. Adeno-papillomatous formation without boundary or capsule. $\times 100$.



4

5



6



8



9



FIGS. 5 TO 9

12 to 18 days duration inflammatory processes with an abundance of polymorphonuclear leukocytes were the most prominent feature. These inflammatory processes always caused the death of the animal. Here large areas of the lung tissue were destroyed and filled with masses of leukocytes and detritus. Nevertheless there were some animals which showed proliferative changes of the above described nature. In one rat which received injections of urethane in a period of 18 days and died on the twentieth day inflammatory infiltration of the lung was found together with intense proliferation of the epithelial cells of the bronchioli which formed several layers and contained abundant nuclei often in mitosis (Fig. 9). The proliferating cells occasionally grew backwards into the peribronchial tissue. Here fibroblastic reaction was evident.

DISCUSSION

Our observations reveal that 21.4 percent of the young adult rats treated with daily intraperitoneal injections of urethane developed adenoma- and papilloma-like formations in the lungs from 6 to 18 days after the first injection. The majority of the other treated animals without frank adenoma showed hyperplasia of the bronchial mucosa, proliferation and downgrowth of bronchial epithelium, and proliferation of the lining cells of the pulmonary alveoli.

The first changes, which could be observed as early as 48 to 72 hours after the beginning of treatment, were a conspicuous edema around the vessels, and in the pulmonary parenchyma vascular congestion and sometimes hemorrhages, and simultaneously hyperplasia and proliferation of the bronchial epithelium. The epithelial layer of the bronchi was extensively folded and in some places there were papillary processes obstructing the lumen. Here and there mitoses were seen. As early as 4 to 13 days after the beginning of treatment, besides a proliferation of all epithelial elements—bronchial as well as alveolar—tumor-like formations were seen. They were of two main types, as described also by Orr (9, 10), namely adenomatous and tubulo-papillomatous structures. In some animals both types as well as transitional stages were observed.

The adenomatous type was represented by alveoli lined with cubical or high columnar epithelium, sometimes secreting mucus. Several such for-

mations lay together, sometimes surrounded by fibroblastic tissue. The picture recalled that described by Cowdry (1) as "Jagziekte" and by Dungal (3) as epizootic adenomatosis in sheep. A similar process was reported in man by Taft and Nickerson (15) as "Pulmonary mucous hyperplasia." Willis and Brutsaert (17) noted formations of this kind in guinea pigs treated with silica dust.

The second type showed predominantly tubulo-papillomatous formations. The papillomatous villi consisted of a capillary loop with a layer of connective tissue, covered by epithelium, sometimes ciliated. These formations were sometimes bordered for short stretches by a thin muscularis layer, whereas at other points no demarcation could be seen and there was backward growth into the surrounding lung tissue. These structures were somewhat similar to the papillomatous described by Magnus (6) in the lungs of mice treated with 1,2,5,6-dibenzanthracene. The surrounding lung tissue was mostly collapsed and contained spindle cells, round cells, and a few polymorphs.

Regarding the histogenesis of the process, it seems that the first stage is the edema of the lung tissue followed very early by a simultaneous proliferation of the epithelial and mesenchymal elements. The proliferation of the epithelium begins in the larger bronchi, descends rapidly into the respiratory bronchioles and alveolar ducts, and eventually involves the lining cells of the alveoli. The proliferation of the mesenchymal cells seems to be a sequel to the edema.

Orr (9, 10) pointed out that the proliferation of lung epithelium after treatment with urethane is the result of a chronic inflammatory process. However, he noted the paucity of leukocytes. His earliest observations were on mice which died 76 days after the beginning of the experiment. Selbie and Thackray pointed out that the tumors observed by them in CBA mice after treatment with urethane are not preceded by pneumonia changes. Our observations suggest that the alveoli become partly filled with edematous fluid as a sequel to the continuous effect of urethane on the vascular system, and parts of the lungs are collapsed. Simultaneously proliferation of all cell elements occurs. Whether

FIG. 5.—Same rat. Numerous small, tubular formations and proliferating cells adjacent to one large, cystadenomatous structure. $\times 140$.

FIG. 6.—Same rat. Alveoli invested by a layer of high cylindrical cells. $\times 200$.

FIG. 7.—Rat 333, treated with 6 intraperitoneal injections of urethane over a period of 6 consecutive days and killed on the seventh

day. Alveolar ducts and alveoli covered by high cylindrical cells. $\times 205$.

FIG. 8.—Same rat. Group of adenomatous formations surrounded by fibroblastic tissue. $\times 100$.

FIG. 9.—Rat 536, treated with 14 intraperitoneal injections of urethane over a period of 18 days. The animal died after 20 days. Bronchiolar mucosa showing proliferation of the epithelial cells. Some cells in mitosis. $\times 580$.

this cell proliferation is due to the influence of urethane or to some virus present in the lungs of the animals and activated by urethane, is not known. Investigations are in progress on the fate of the described epithelial proliferations, to determine whether they are permanent or reversible. The minor incidence of adenomatous formations in lungs of rats treated for 12 to 18 days with daily injections of urethane may be due to the destruction of large areas of the lung tissue by extensive inflammatory processes with abundant polymorphonuclear leukocytic infiltration.

The problem of the rapid development of the observed tumor-like formations remains. There is a very slight possibility that these changes in the lungs of the experimental animals might have occurred spontaneously, though we were never able to observe changes of a similar nature in the lungs of control rats or of hundreds of rats of the same stock treated with other substances. Dr. E. J. Farris, Executive Director and Associate in Anatomy, The Wistar Institute, Philadelphia, informed us that he has never observed these changes in large colonies of normal rats.

The changes seen by us are only microscopic foci and our experience with tissue cultures of lungs (11) leads us to believe that proliferation of epithelial cells can be very rapid. There are also some reports of rapid proliferation of epithelium in the lungs of experimental animals. Straub (14) observed proliferation of epithelial cells of the respiratory bronchioles and the alveoli with numerous mitoses in the lungs of mice infected with influenza virus as early as 5 days after the infection. Thornton and Adams (16) saw changes in the bronchial epithelium from typical to transitional epithelium as early as 5 to 7 days following application of benzopyrene in rats. Smith and Rous (12) observed adenomata in infant mice, when the mother had been treated with urethane before parturition; the adenomata were found 10 days after the first injection into the mother. The authors think that the rapid development of the tumors is due to "conditions implicit in the youth" of the cells "which already possess a natural tendency to divide." Our observations show that rapid development can also occur in young adult rats. In both cases the reduced respiration either in the days before birth or as a consequence of the deep narcosis induced by urethane may favor the proliferation of epithelium.

It must be assumed that urethane has a growth-promoting effect on the epithelial cells of the lung. On the other hand, urethane is known to be a capillary poison, augmenting the permeability of the capillary wall. The lung tissue, filled with extravasated plasma, offers a very suitable environ-

ment for the proliferation of cells as is known from many pathological conditions. Possibly this combined effect facilitates the rapid development of tumors in the lungs.

SUMMARY

Proliferation of the epithelial cells of the lung was observed in rats treated with daily intraperitoneal injections of urethane. Adenomatous and papillomatous formations were microscopically observed as early as 4 to 13 days after the first injection. The histology of these early changes is described and their histogenesis discussed.

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Further Studies of the Immunological Properties of Polysaccharides from *Serratia marcescens* (*Bacillus prodigiosus*)

III. Passive Immunization Against the Lethal Activity of the Polysaccharides with Fractions of Mouse Antiserum Elicited by a Single Injection of Polysaccharide*

HUGH J. CREECH, PH.D., AND REED F. HANKWITZ, JR., B.S.

(From the Lankenau Hospital Research Institute and The Institute for Cancer Research, Philadelphia 30, Pennsylvania)

In previous work (1 to 4), it was demonstrated that mice could be protected against the lethal action of polysaccharides from *Serratia marcescens* by procedures of passive immunization which, in certain instances, did not seem to interfere with the tumor-necrotizing action. Moderate amounts of the serum globulin fractions from rabbits given multiple injections of the polysaccharides afforded mice substantial protection only against the lethal action of polysaccharides from the homologous strain of organism. When greater amounts of the rabbit globulin fractions were used, it was noted that the mice were protected also against the serologically unrelated polysaccharides from a different strain of organism. Globulin fractions from sera of mice given a single injection of polysaccharide were found to afford mice bearing sarcoma 37 considerable protection against the homologous polysaccharide and also, in one test, against the heterologous polysaccharide (3). Active immunization of the mouse with the polysaccharides seemed to confer an entirely non-specific protection (3). Because of the indications of lack of strain specificity in the mouse compared with the rabbit, it was considered of interest to study in greater detail the effects of passive immunization with mouse antisera. As the supplies of these particular preparations of polysaccharides are nearly exhausted and since our plans for future investigations with new preparations are different from those already described, it seemed advisable to present the following results as a concluding report of this phase of the study.

* This research was supported in part by an American Cancer Society grant recommended by the Committee on Growth of the National Research Council, and by a grant from the National Institute of Health.

EXPERIMENTAL

The preparations of the polysaccharide-lipid complex used in this work were P-10, P-10a, and P-20 from the 724 strain of *Serratia marcescens* and P-3.M and P-3.Ls from the G.W. strain. As before, these materials were supplied to us by Dr. M. J. Shear of the Chemotherapy Section of the National Cancer Institute.

Except for one special experiment, 7- to 8-week old Swiss mice from our stock colony were injected intraperitoneally with 100 γ of a preparation of polysaccharide and exsanguinated by heart puncture either 3 or 14 days later. The serum was fractionated with ammonium sulfate into the globulin and albumin components. After dialysis, determination of the protein content by micro Kjeldahl analyses, and adjustment of the protein level to 10 to 15 mg. per cc., a 1-cc. quantity of the protein in physiologic saline was injected intraperitoneally into 7- to 8-week old Swiss mice bearing sarcoma 37 which had grown to a diameter of 12 to 15 mm. Three hours later, these mice were injected intraperitoneally with 0.5 cc. of a saline solution of the amount of polysaccharide which ordinarily kills about 70 per cent of a group of tumor-bearing Swiss mice. Control groups of mice injected only with the polysaccharide, and groups injected with either bovine serum albumin or the globulin fraction from normal Swiss mice prior to the administration of polysaccharide, were included in the experimental series.

RESULTS

The observations noted in the series of tests have been compiled and summarized in the accompanying table. When 600 γ of the polysaccharide P-10a (724 strain) was administered to a

total of 85 tumor-bearing Swiss mice, it was found that 73 per cent of the mice died within 24 hours and 81 per cent died within 96 hours (line 1). This 96-hour mortality rate was assigned a ratio of 1 in the last column of the table. Prior injection of the mice with either bovine serum albumin or with the globulin fraction from blood of normal 8-week old Swiss mice at ratios of protein to polysaccharide of 16 to 25 afforded them little protection against the lethal action of the polysaccharide (lines 2 and 3). A study was also made of the influence of a passive transfer of the serum albumin fraction from mice given a single 100- γ injection of P-10. This fraction isolated from blood obtained 3 days

reduction in the mortality rate was noted following the use of the globulin fraction from sera of mice obtained 10 days after the injection of 250 γ and 400 γ of P-20, spaced 7 days apart, into Swiss mice bearing sarcoma 37 (glob. S/P-20, line 11) and after injection of 100 γ and 800 γ of P-20, spaced 4 days apart, into normal Swiss mice (glob. N/P-20, line 12).

These observations were confirmed in a separate series of tests in which a 500- γ amount of P-10a was used as the lethal dose. Of the serum globulins obtained from the mice 3 days after a single injection of polysaccharide, it is seen that the globulin fraction of antiserum elicited by P-10 was highly

TABLE 1

PASSIVE IMMUNIZATION OF SWISS MICE BEARING SARCOMA 37 USING FRACTIONS OF SERA FROM SWISS MICE INJECTED WITH POLYSACCHARIDE

LINE	MOUSE SERUM	PROTEIN	POLY.	AMT. IN γ	PROTEIN/POLY.	RATIO:	NO. OF MICE	PER CENT DEATHS In 24 hrs. In 96 hrs.	PER CENT DEATHS In 96 hrs.	EXPT./CONTROL	RATIO (96 HR. MORTALITY):
1	Glob./normal	"	P-10a	600	0		85	73	81		1.0
2	Bov. ser. alb.	"		"	16-25		46	50	61		0.75
3	Alb. 3/P-10	"		"	20		34	47	65		0.80
4	Alb. 14/P-10	"		"	"		18	50	56		0.69
5	Glob. 3/P-10	"		"	"		21	57	76		0.94
6	Glob. 14/P-10	"		"	16-25		23	17	26		0.32
7	Glob. 3/P-20	"		"	"		24	17	21		0.26
8	Glob. 14/P-20	"		"	"		28	25	43		0.53
9	Glob. 3/P-3.M	"		"	"		31	19	19		0.93
10	Glob. S/P-20	"		"	"		42	43	48		0.59
11	Glob. N/P-20	"		"	"		20	10	10		0.12
12	"	"		500	0		16	31	50		0.19
13	Glob. 3/P-10	"		"	20		9	0	0		1.0
14	Glob. 3/P-20	"		"	20		8	0	25		0.5
15	Glob. 3/P-3.M	"		"	20		16	38	50		1.0
16	P-3.Ls			300	0		30	63	77		1.0
17	Glob. 3/P-3.M	"		"	50		14	29	29		0.38
18	Glob. 3/P-3.M	"		"	100		10	30	30		0.39
19	Glob. 14/P-10	"		"	50		8	50	63		0.82
20	Glob. 14/P-20	"		"	50		9	67	67		0.87

after the injection exerted a slight protective action (line 4); when obtained at 14 days, the albumin fraction was ineffective against the lethal action of P-10a (line 5).

From lines 6 and 7, it is seen that the injection of the globulin fraction (glob. 3/P-10 and glob. 14/P-10) from sera of mice obtained either 3 or 14 days after a single injection of P-10, had a pronounced effect in decreasing the lethal activity of the polysaccharide P-10a. The globulin fraction of sera toward a different preparation (P-20) of polysaccharide from the same strain of organism appeared to be less protective when obtained on the third day than on the fourteenth day (lines 8 and 9). Prior injection with the globulin fraction from sera of mice obtained 3 days after a single 100- γ injection of the P-3.M polysaccharide (G.W. strain) afforded the mice slight protection against the lethal action of P-10a (line 10). An extensive

effective and that by P-20 was only moderately effective, whereas the globulins from sera of mice injected with P-3.M showed no protective action against P-10a (lines 13 to 16).

In another series of tests in which 300 γ of the polysaccharide P-3.Ls from the G.W. strain was employed as the lethal dose, it was observed that the globulin fraction of mouse sera obtained 3 days after a single injection of 100 γ of P-3.M conferred a substantial degree of protection on the mice (lines 18 and 19). The globulin fraction of mouse sera obtained 14 days after a single injection of 100 γ of P-10 or P-20 had no significant effect (lines 20 and 21).

These results substantiate and extend our earlier findings (3, 4) that protective antibodies were elicited rapidly in the mouse by a single injection of polysaccharide. They also demonstrate that globulin fractions obtained from mouse sera following a

single injection of P-10 polysaccharide afforded mice a high degree of protection against the lethal action of that polysaccharide at relatively low globulin to polysaccharide ratios of 16 to 25. Similar extents of protection against P-10 were noted previously only with globulin to polysaccharide ratios of about 100 when the γ -globulin fractions of rabbit antisera elicited by a series of injections of P-10 were employed (3).

In addition, the serum globulin fractions from mice given a single injection of the polysaccharides exhibited a degree of specificity similar to that observed with globulin fractions from rabbit antisera elicited by multiple injections (2, 3). Thus, at low globulin to polysaccharide ratios, there was no significant cross protection between the polysaccharides from the 2 strains (lines 10, 16, 20, and 21) whereas at higher ratios of about 100, there was cross protection (3, Table 3) with mouse as well as rabbit globulin fractions from antisera toward the polysaccharides (2, 3).

SUMMARY

It has been found that passive immunization with relatively small amounts of the globulin frac-

tions obtained from sera of mice either 3 or 14 days after a single injection of polysaccharides from *Serratia marcescens* afforded mice bearing sarcoma 37 pronounced protection against the lethal action of the homologous polysaccharide but not against that of the polysaccharides from a different strain of the organism.

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American Association for Cancer Research, Inc.

40th Annual Meeting

Hotel Fort Shelby, Detroit, Michigan

April 16 and 17, 1949

Proceedings of Scientific Sessions

A SEROLOGICAL FLOCCULATION REACTION AS AN INDICATION OF MALIGNANCY. H. S. PENN. (Department of Zoology, University of California, Los Angeles, Cal.)

When the serum of a patient suffering from malignancy is mixed with the unsaponifiable fraction obtained from human cancerous liver, there is noted in a majority of instances (between 80 to 98 per cent) a flocculation characterized by the appearance of particles, and partial or complete clearance of the opaque mixture. This flocculation occurs only rarely (about 1 per cent) when the serum of healthy individuals is employed, and to a limited extent (20 to 25 per cent) in other pathological conditions. The phenomenon is not observed when the lipoid of non-cancerous livers is used as an antigen.

This investigation was based on the hypothesis of the formation of specific antibodies in response to an endogenous carcinogen, presumably steroid in nature. A complete description of the preparation of the antigen and the method of application is given. Approximately 3,000 sera were tested. Of these, 312 biopsy-proven positive sera were submitted as unknowns. Correct diagnoses were made in 259 cases (83 per cent). The accuracy of the flocculation reaction seems to vary with different malignancies, cancer of the skin being the least reliable (about 70 per cent), whereas bronchogenic carcinoma has given about 98 per cent correct diagnoses.

HETEROLOGOUS OCULAR TRANSPLANTATION AS A PRACTICAL TEST FOR CANCER.

JOHN A. SCHILLING, ALBERT C. SNELL, JR., and BENEDICT V. FAVATA. (Department of Surgery, The University of Rochester School of Medicine and Dentistry, Rochester, N.Y.)

Carefully selected fragments from 36 proven human cancers were transplanted into the anterior chamber of 368 eyes in 218 guinea pigs. Growth occurred in eight of the 36 cases in 29 eyes of 20 guinea pigs. Growth occurred in 15 of 19 of these cases when the same human tissue was studied simultaneously by routine tissue culture *in vitro*. Growth was delayed as long as four months after intraocular transplantation, necessitating an arbitrary period of at least six months' observation before considering the result final. When growth occurs after

transplantation into the anterior chamber of the eye of a heterologous host, according to well defined criteria, it is definite evidence of a high degree of autonomy and may be helpful in gaining insight into the growth potential of a given neoplasm. The method is not reliable for the clinical diagnosis of cancer because of the high incidence of failures (78 per cent) of growth in proven malignancy.

THE TISSUE TRANSPLANT TECHNIC AS A MEANS FOR TESTING MATERIALS FOR CARCINOGENIC ACTION. WILLIAM E. SMITH, M.D. (Sloan-Kettering Institute for Cancer Research, New York 21, N.Y.)

Induction of cancers from epithelial tissues of mouse embryos has been previously described. The method has been to transplant pieces of selected organs together with methylcholanthrene into adult hosts of an inbred strain of mice. Neoplastic potentialities of various tissues were thus explored.

The method has now been extended with a view to employing it as a means of testing materials for carcinogenic action. Olive oil, the solvent used in previous tests, has been replaced by tricaprylin. Cancers have been readily induced from fragments of mouse embryo skin transplanted with equimolar (0.02M) solutions of 20-methylcholanthrene, 3,4-benzopyrene or 1,2,5,6-dibenzanthracene. Each of these solutions elicited squamous cell cancers or malignant papillomas at practically every implant site: within 45 to 73 days with methylcholanthrene, within 108 to 175 days with the other two carcinogens.

To adapt the method for study of weak carcinogens, experiments were carried out with a barely effective (0.01M) concentration of dibenzanthracene with and without addition of 2 per cent Scharlach R and 0.1 per cent croton oil. These latter substances, not in themselves carcinogenic, increased the yield of cancers.

This technic is being employed for assaying carcinogenicity of chemicals available only in small amounts. Tests have also been carried out with tissues available in only small amounts. A transplantable carcinoma has been induced from fragments of thyroid tissue taken from a young adult mouse and transplanted with methylcholanthrene.

FACTORS INFLUENCING THE STABILITY OF CULTURED STRAINS OF NORMAL AND MALIGNANT CELLS. GEORGE O. GEY and FREDERIK B. BANG (by invitation). (The Johns Hopkins University Medical School, Baltimore 5, Md.)

Prolonged cultivation of normal and malignant cells has revealed a wide degree of variation occurring within some of the stocks while others remain fairly stable. Many of the normal and malignant strains to be described have been under cultivation for from 10 to 18 years. Some of the normal strains have become permanently altered *in vitro* and others have become malignant. Some malignant strains of host origin show great uniformity in their physiological and morphological characteristics. Others have shown profound changes after prolonged cultivation. The factors which presumably can alter the stability of cells *in vitro* will be discussed. They include viruses, hormones, and nutritive conditions. The study also emphasizes the comparative importance of homologous and heterologous media for the maintenance of some types of cells, and for the study of virus-host cell relationships.

Media sterilized with fast electrons (Capieitron) or with ultraviolet (2537) have been successfully used for cell cultivation in order to eliminate virus contaminants. This method may prove important for the study of specific viruses grown with cells and for studies with the electron microscope.

A STUDY IN VITRO OF A STRAIN OF LYMPHOBlast-LIKE CELLS, MB13, DERIVED FROM MOUSE LYMPHOSARCOMA MB (T86157). WILLEMINA M. DE BRUYN. (Division of Cell Physiology, Department of Surgery, The Johns Hopkins Hospital and School of Medicine, Baltimore 5, Md.) (Introduced by George O. Gey.)

Previous investigations have shown that the malignant lymphoblasts of lymphosarcoma MB could be maintained in continuous roller tube culture indefinitely, but only in the presence of growing mesenchymal cells. A few cultured cells can produce a tumor and kill in 16 days.

Cultivation of malignant lymphoblasts without fibroblasts was attempted by varying the media and the temperature. In several tubes in one experiment the fibroblasts died out, leaving only masses of active lymphoblast-like cells. They had been grown in chicken plasma, human cord serum, bovine embryo extract, and balanced salt solution. A strain of lymphoblast-like cells, MB13, was developed from one of these tubes. These cells vary much in size and shape. They now grow without fibroblasts and without plasma. They need embryo extract and grow best when only part of the medium is replenished with fresh fluid. Four months after the change in the cells had taken place, they still produced a tumor. From then on all injections were unsuccessful. Since these cells had been cultivated in an entirely heterologous medium for several months, experiments are under way to find out if the MB13 cells have lost their ability to grow in the mouse. The following experiments were attempted:

a. Re-establishment of mouse fibroblasts and MB13 cells in a heterologous medium.

b. Cultivation of MB13 cells, but without fibroblasts, in a medium with mouse embryo extract replacing bovine embryo extract.

To date lymphoblast-like cells, MB13, have failed to grow with mouse mesenchymal cells, whereas they grow equally well in media with mouse embryo extract as in media with bovine embryo extract.

THE EFFECT OF REGENERATION ON THE GROWTH CAPACITY OF RAT LIVER *IN VITRO*. ANDRÉ D. GLINOS. (From the Division of Cell Physiology, Department of Surgery, Johns Hopkins Hospital and Medical School, Baltimore, Md.)

The present work is the first part of a study aiming to investigate further the relationship between liver regeneration and carcinogenesis with *p*-dimethylaminobenzene. The roller tube tissue culture method of Gey was used in devising a test for the growth capacity *in vitro* of normal rat liver and the effect of regeneration upon it. The following experimental procedure was used on each animal: a) partial hepatectomy, b) immediate explantation of part of the removed median lobe, c) explantation, 72 hours post-operatively, of part of the remaining right lateral lobe.

It was found that the growth capacity of normal rat liver is an inverse proportional function of the age of the animal. In the case of young rats, 4 to 8 weeks old, 10 out of 10 or 100 per cent of the explanted livers grew *in vitro*; in adult rats, 4 to 8 months old, 5 out of 11 or 45 per cent; in old rats, 18 to 24 months old, 1 out of 12 or 8 per cent.

Regeneration had a marked effect on this relationship between age and growth capacity *in vitro*. In the young rats 7 out of 7 explanted regenerating livers grew *in vitro*; in the adults 11 out of 11; in the old rats 8 out of 8. Thus the growth capacity of regenerating liver, 72 hours after partial hepatectomy, was 100 per cent throughout the age groups and therefore independent of the age of the animal.

Differences in the morphology and organization of the colonies of normal and regenerating liver and their relationship to age have also been established.

CERTAIN RECENTLY DEVELOPED TISSUE CULTURE PROCEDURES APPLICABLE TO STUDY OF THE NUTRITION OF NORMAL AND MALIGNANT CELLS. W. R. EARLE, K. K. SANFORD, V. J. EVANS, E. L. SCHILLING (by invitation), and G. D. LIKELY (by invitation). (National Cancer Institute, Bethesda 14, Md.)

This paper summarizes a group of recently developed procedures by which a strain of sarcoma cells has been grown from a single cell, and by which cells may be grown in tissue culture under sheets of perforated cellophane instead of embedded in a plasma matrix of unknown and variable composition.

When so grown the cells can be scraped off from the cellophane and made up as a cell suspension which can be used to plant up to 200 or more replicate cultures

from one uniform inoculum suspension. By a special hemocytometer technique the actual number of cell nuclei in each unit volume of the original cell suspension planted can be determined. By sacrificing individual cultures from a series, changes in the number of nuclei present may be accurately followed. The procedures seem particularly well adapted to proliferation studies of the nutrition of the malignant cell and of the normal cell from which it arose.

In the instance of rapidly growing cell strains, using these cellophane procedures, dense sheets of cells covering areas of 70 square centimeters can be obtained in a few days. While the largest sheet so far obtained had an area of only about 190 square centimeters, work is now in progress to further adapt the method for practicable routine growth of far larger cultures.

THE LETHAL MUTATION RATE IN *DROSOPHILA MELANOGASTER* FOLLOWING THE ADMINISTRATION OF 20-METHYLCHOLANTHRENE AND METHYL-BIS(BETACHLOROETHYL)AMINE HYDROCHLORIDE.*

WALTER J. BURDETTE. (School of Medicine, Louisiana State University, New Orleans, La.)

There is some evidence that the carcinogen, 20-methylcholanthrene, is also mutagenic in mice. Since it is highly important to know if these two properties are related, it is desirable to test the mutagenic activity of carcinogens in a form which is more suitable for the detection of such changes. Using the Muller-5 and Oregon-R stocks of *Drosophila melanogaster* tests were made for lethals on the x-chromosome. The females were treated with 20-methylcholanthrene in sesame oil by the vaginal douche technic. This was continued serially for 12 generations during a period of 148 days. The wild type chromosomes tested each successive generation had therefore been treated from 1 to 12 times. Moreover for the last 10 generations the *sc⁸ w^a* B chromosomes treated twice were also tested. Three lethals on the *sc⁸ w^a* B chromosome, one visible mutation to *y*, and one instance of non-disjunction were found among the 4,660 chromosomes tested. It is apparent that 20-methylcholanthrene was not mutagenic in these studies although the same material was carcinogenic for C3H mice. On the other hand the mutation rate for these stocks was increased simply by dropping methyl-bis(betachloroethyl)amine hydrochloride on the abdomen. In one such experiment there were 15 lethals among 2,494 chromosomes tested. The results with methylcholanthrene may be explained either by assuming it is not mutagenic or that it is strain limited or not effective by the route and concentration used. This should make one cautious in interpreting isolated instances of mutations as necessarily being due to concomitant treatment with carcinogen.

POLYCYTHEMIA ASSOCIATED WITH A TRANSPLANTABLE LUTEOMA. RAYMOND G. GOTTSCHALK and JACOB FURTH. (Veterans Administration Hospital and Southwestern Medical College, Dallas, Texas)

* This investigation was supported by a grant from the National Cancer Institute, U.S. Public Health Service.

Repeated blood volume and hematocrit determinations were made on mice by means of a microtechnic requiring less than 0.1 cc. of blood. The occurrence of hypervolemia with grafted granulosa cell tumors has been confirmed. The increase of blood volume is mainly due to an increase of the plasma volume, with little variation of the total volume of red cells, and with a relative anemia. Transplantable luteomas are associated with polycythemia, the number of red cells rising as much as 50 per cent; the hematocrit values are elevated by as much as 30 per cent. Luteoma bearing mice generally have but a mild hypervolemia due to the increase of erythrocytes, the plasma volume remaining about constant. The most severe polycythemia was found in cases of intrahepatic graft of the tumor.

These observations recall data indicating that estrogens decrease and androgens and adrenal cortical hormones increase the number of red cells. The granulosa cell tumors produce estrogens, while the luteomas are masculinizing. The hematologic changes secondary to these ovarian tumors seem related to the influence of sex hormones on erythropoiesis. The mice with transplanted luteomas show most of the elements of Cushing's syndrome, including polycythemia; this syndrome is also associated with the so-called adrenal rest tumors of the ovary in human patients.

CONTRASTING EFFECTS OF FOLIC ANTAGONISTS AND NITROGEN MUSTARDS ON LEUKEMIC CELLS *IN VIVO*.* J. H. BURCHENAL, † M. A. CREMER, and B. S. WILLIAMS (Introduced by C. P. ROADS.) (Section on Mouse Leukemia of the Division of Experimental Chemotherapy, Sloan-Kettering Institute for Cancer Research, New York 21, N.Y.)

A secondary screening technique has been used to study compounds which have previously shown the ability to prolong the survival of mice with transmitted leukemia. In this method advantage has been taken of the delayed toxicity even at high doses of certain of these agents in examining the short term effects of supra-lethal doses in inactivating leukemic cells *in vivo*.

Donor mice having enlarged spleens from a generalized leukemia or with localized tumors from injection of the leukemic suspension by the subcutaneous route were injected intraperitoneally with various multiples of the LD₅₀ dose of a given compound. Two hours later or immediately after death if the drug at very high doses was lethal in less than this time, the spleens or tumors were removed under aseptic precautions, minced in saline, and injected intraperitoneally into recipient mice of the same inbred stock for bioassay. At least 2 donor mice were used at each level and the leukemic suspension from each donor was bioassayed into 4 mice.

With the nitrogen mustards tested, a cytotoxic or inactivating dose was found to occur in the range between

* This investigation was supported (in part) by a research grant from The National Cancer Institute of The National Institutes of Health, U.S. Public Health Service, and (in part) by a research grant from The American Cancer Society.

† Fellow of The American Cancer Society, recommended by the Committee on Growth of The National Research Council.

four and ten times the LD₅₀. With 4-amino-pteroyl glutamic acid, 4-amino-N10-methyl-pteroyl glutamic acid, and 2,6-diaminopurine, however, the bioassays were positive up to and including one hundred times the LD₅₀. Even after exposure for 48 hours to 20 times the LD₅₀, no inactivating effect was seen.

THE INFLUENCE OF PROGESTERONE ON MAMMARY TUMORS INDUCED IN RATS BY ACETAMINOFLUORENE. A. CANTAROW, K. E. PASKIKIS, and J. STASNEY. (Jefferson Medical College, Philadelphia 7, Pa.)

Data previously reported from this laboratory indicated that administration of progesterone greatly increased the incidence of mammary carcinoma in female Sherman and Wistar rats receiving 2-acetaminofluorene. Estradiol was apparently without influence in intact rats. The possibility was suggested that whereas small amounts of estrogen are probably necessary for the development of breast cancer in rats receiving AAF, the quantity of progesterone may be the limiting factor in this connection. The present experiments represent an attempt to test this hypothesis.

(a) Progesterone was administered to castrate male and female rats, (b) progesterone and estradiol to intact males, (c) progesterone and estradiol to castrate males and females, (d) progesterone and estradiol to intact females, and (e) progesterone and testosterone to intact males.

No breast tumors developed in progesterone-treated intact males and castrate males and females, nor in progesterone-testosterone-treated males. One tumor developed in 10 intact males receiving estradiol and progesterone. Two tumors appeared in 33 castrate males receiving estradiol and progesterone. Four tumors appeared in 20 castrate females receiving estradiol and progesterone. One tumor appeared in 24 intact female rats receiving estradiol and progesterone. This is in sharp contrast to the 85 per cent incidence of breast tumors in such rats receiving progesterone alone and the 25 per cent incidence in intact females receiving no hormone. The significance of these observations is discussed.

STEROID EXCRETION IN GASTRIC CANCER.

K. DOBRINGER, S. LIEBERMAN (by invitation), J. ABELS (by invitation), F. HOMBURGER, E. C. REIFENSTEIN, JR., AND C. P. RHoads. (Sloan-Kettering Institute for Cancer Research, New York 21, N.Y.)

Gastric cancer is one of the most important types of neoplastic disease not only because of its high incidence but also because early diagnosis presents so many difficulties. These considerations led us to investigate the steroid excretion pattern in 2 men and 2 women suffering from advanced gastric cancer. In all of these patients a metabolite of adrenal cortical origin, 11β-hydroxy-etiocholanolone was identified. This same compound was previously found in a significant number of patients with neoplastic disease and was rarely present in the urine of normal individuals (Dobriner, Lieberman, and Rhoads, *Cancer Research*, 7:711, 1947). Two other compounds of adrenal cortical origin, 11β-hydroxy-

androsterone and 11-ketoetiocholanolone, were found in the urine of all 4 gastric cancer patients. These substances are regular constituents of the urine but the amount excreted by the gastric cancer patients was smaller than that usually present in normal subjects. Androsterone and etiocholanolone, both constituents of the urine of normal people were either absent or present in very small amount in the gastric cancer patients. The pattern of ketosteroid excretion in gastric cancer resembles those obtained in prostatic cancer. The clinical significance of steroid metabolism in relation to adrenal cortical function will be discussed.

THE EFFECT OF TESTOSTERONE PROPIONATE THERAPY ON THE EXCRETION OF HORMONES IN PATIENTS WITH METASTATIC BREAST CARCINOMA.* ALBERT SEGALOFF, RICHARD L. COPPEDGE (by invitation, and J. V. SCULOSSE (by invitation). (Departments of Medicine and Physiology, Tulane University, the Alton Ochsner Medical Foundation, and the Charity Hospital of Louisiana, New Orleans, La.)

Seven patients have been studied to date in whom it has been possible to obtain excretion studies before and during testosterone propionate therapy for carcinoma of the breast with bone metastases. Six of the 7 patients showed the expected increase in 17 keto-steroids while on testosterone propionate therapy. Androgen bioassays have been completed on one patient and this patient also showed an increase in androgen excretion. One patient had no detectable gonadotrophic hormone before therapy, probably due to her advanced cachetic state. The others showed normal or elevated urinary gonadotrophic hormone and showed the expected decrease in its excretion while on therapy.

Cortin excretion was measured by its ability to deposit hepatic glycogen in adrenalectomized mice. This was studied in 6 patients, 3 of whom showed a marked increase in excretion while on therapy. The other 3 showed little or no change. The urinary excretion of lactogenic hormone was studied in 4 patients, 3 of the 4 showing an increase in excretion.

Clinically, 5 of the 7 patients had a marked improvement in course while on testosterone therapy, most of them gaining weight and having freedom from pain. The other 2 apparently had little or no effect on the course of their disease as the result of therapy with testosterone propionate. The testosterone propionate therapy was given as intramuscular injections of 100 mg. every other day.

INFLUENCE OF AGE OF HOST AND OF OVARIES ON TUMORIGENESIS IN INTRASPLENIC AND INTRAPANCREATIC OVARIAN GRAFTS. W. U. GARDNER and M. H. LI (by invitation). (Department of Anatomy, Yale University School of Medicine, New Haven, Conn.)

Four groups of experiments were undertaken to determine the effect of age of the ovary and age of the host

* This investigation was supported by a research grant from the National Cancer Institute of the National Institute of Health, U.S. Public Health Service.

on experimental ovarian tumorigenesis. (a) Ovaries from young mice were transplanted intrasplenically into young mice. (b) Old mice (204 to 370 days) were castrated and ovaries from animals of similar ages implanted intrasplenically. (c) Ovaries from old mice (304 to 491 days) were transplanted intrasplenically or intrapancreatically into young castrated mice.

The age of the host was more important than the age of the donor; hosts showed a high incidence of ovarian tumors, granulosa cell and luteomas, irrespective of the age of the ovarian transplant at the time of grafting. Both intrasplenic and intrapancreatic transplants of ovaries into castrated mice became tumorous. Mice of all inbred strains used, one not studied previously in such experiments, showed similar responses. The tumors arising from the transplants of old ovaries were not unlike those arising from transplants of young ovaries.

These experiments indicate that ovarian granulosa cell tumors and luteomas can arise from the aged ovary and that the humoral environment of the younger animal is more favorable.

SEX HORMONE SECRETION BY ADRENAL CORTICAL TUMORS OF MICE. MARIELLA J. FRANTZ (by invitation), and ARTHUR KIRSCHBAUM. (Department of Anatomy, University of Minnesota Medical School, Minneapolis, Minn.)

The type of sex hormones secretion in gonadectomized mice bearing cortical adenomas was determined by histologic study of submaxillary glands, renal corpuscles, and reproductive tracts. Animals of both sexes of the NH and Bagg albino strains, and females of the C3H, CBA and Strong A strains were studied.

Adenomas which secreted only estrogenic hormone were present in NH mice 70 or more days following gonadectomy. Similar tumors (10 months post-operatively) of the Bagg albino stock secreted primarily androgenic hormone. The seminal vesicles of most males castrated 2 years before autopsy were developed and secreting; submaxillary glands and kidneys were masculinized. Ten months following ovariectomy submaxillary glands of CBA and C3H females were masculinized, whereas the vaginal epithelium was cornified, indicating simultaneous secretion of estrogen and androgen. In the Strong A strain, the reproductive tract was atrophic, although submaxillary glands were male, 14 to 16 months following ovariectomy, indicating secretion of only androgen.

Hormonal secretion of cortical tumors was characteristic for the strain. The earlier tumors developed following gonadectomy, the greater the tendency towards estrogenic activity. Where both sex hormones were secreted simultaneously 10 months following gonadectomy as in the CBA and C3H strains, primarily estrogenic activity was detected from 6 to 8 months post-operatively. In the Strong A strain where the latent period of tumor formation was relatively long, cortical tumors demonstrated only androgenic activity.

Cessation of gonadal endocrine secretion was not essential for the development of spontaneous cortical tumors in both sexes of the NH and Bagg albino strains.

LEYDIG CELL TUMORS INDUCED EXPERIMENTALLY IN THE RAT. GRAY H. TWOMBLY, DORIS MEISEL (by invitation), AND ARTHUR PURDY STOUT. (College of Physicians and Surgeons, Columbia University, New York 32, N.Y.)

Fifty young adult castrated female rats were treated by the transplantation of a testis into the spleen. The testes were taken from day old rats and the implantation was done beneath the capsule of the spleen with a trochar. A similar group of 50 male castrates and a control series of 25 normal males were prepared in the same way. The animals died or were sacrificed after 240 to 450 days. In the castrated females 20 Leydig cell tumors were found; the largest was 5 em. in diameter. Sixteen tumors occurred in the 50 castrated males. While histologically some of these tumors appeared malignant, no metastases were found. In the control males the remains of atrophic testicular tissue could be seen but no Leydig cell tumors occurred. All stages in the development of these neoplasms from Leydig cell hyperplasia through adenoma formation to what appeared to be malignant tumors were easily seen in this material. Some of the tumors are histologically remarkably like human Leydig cell tumors with which we have compared them.

TUMORS OF PITUITARY AND TRACHEA IN MICE AFTER HIGH DOSAGES OF RADIOACTIVE IODINE. AUBREY GORBMAN. (Department of Zoology, Barnard College, Columbia University, New York, N.Y.) (Introduced by E. D. Goldsmith.)

About ten months after single injections of 4 to 50 milliecurie-per-kilogram dosages of I^{131} tumorous enlargements of hypophyses were found, as well as fibrous tumors of the tracheal tunica propria. The hypophyseal growths, as much as 80 times larger than the normal pituitary, contained patches of normal acidophils, and very few normal basophils. Their bulk was made up of enlarged highly vacuolated cells. Visual and motor disturbances were frequent external manifestations in mice bearing hypophyseal tumors.

The tracheal growths were fibrous in their bulk, but covered with a metaplastic simple or stratified squamous epithelium. This epithelium in some instances was extremely active mitotically. By reduction of the tracheal lumen such tumors resulted in labored respiration and apparently contributed to the death of several animals.

EFFECT OF REVERSED RESTRICTION ON MAMMARY TUMOR INCIDENCE IN OVARIECTOMIZED C3H MICE. CARMEN V. CASAS (by invitation), JOSEPH T. KING (by invitation), and M. B. VISSCHER. (Department of Physiology, University of Minnesota, Minneapolis, Minn.)

The effect of *ad libitum* feeding and caloric restriction on the incidence of mammary tumors in castrate G3H female mice has been tested by reversing type of feeding at various intervals as indicated below. The details of the diet and housing conditions have been published previously by Visscher *et al.* The restricted mice received normal amounts of protein, vitamins and min-

erals. Ovariectomy was performed at weaning and the mice were immediately placed on the diet indicated. The number of mice in each group and the number of tumors occurring through the fourteenth month are shown below.

Feeding	Tumors
Full-fed 14 mos.	15/20
" " 4 mos., then restricted	5/10
" " 3 mos., " "	3/10
" " 1 mo., " "	0/10
Restricted 14 mos.	1/10
" " 7 mos., then full fed	3/10
" " 4 mos., " "	3/10
" " 2 mos., " "	3/10
" 1 mo., " "	6/10

ISOLATION, PROPERTIES AND CHEMISTRY OF ALPHA AND BETA-PELTATIN. J. L. HARTWELL (by invitation), and W. E. DETTY (by invitation). (Chemotherapy Section, National Cancer Institute, Bethesda 14, Md.)

Podophyllin (N.F.) has been shown (Hartwell and Shear, *Cancer Research*, 7:716, 1947; unpublished work of Leiter *et al.*) to contain at least 3 pure, colorless, crystalline components which together account for the major part of the tumor-damaging activity of the whole drug. Of these, podophyllotoxin is well-known while *alpha* and *beta*-peltatin are of recent discovery (Hartwell, *J. Am. Chem. Soc.*, 69:2918, 1947; Hartwell and Detty, *J. Am. Chem. Soc.*, 70:2833, 1948).

These compounds are concentrated in the hexane-insoluble chloroform-soluble fraction which is a brown resin amounting to about 50 per cent of the original drug. This fraction is dissolved in a benzene-alcohol mixture and chromatographed on activated alumina; podophyllotoxin, *beta*-peltatin, and *alpha*-peltatin pass thru the tower in that order. The yields are about 7, 5, and 6 per cent, respectively.

The peltatins form colorless crystals soluble in many organic solvents (including alcohol and chloroform) and in dilute solutions of caustic alkalies, but insoluble in petroleum ether and water. Both melt, without sharp m. p., around 230, and both are levorotatory ($[\alpha]_D^{20} = -115^\circ$ in alcohol).

The peltatins are isomeric with podophyllotoxin, $C_{22}H_{22}O_8$. All three have a methylenedioxy group, and a lactone group. *Alpha*-peltatin has two phenolic hydroxyl and two methoxyl groups, while *beta*-peltatin has one phenolic hydroxyl and three methoxyl groups. Each peltatin gives rise to a diastereoisomer when treated with alkaline reagents, and to two sets of acetates and methyl ethers depending on conditions of formation. With each peltatin one set of derivatives is identical with the corresponding ones prepared from its diastereoisomer.

Evidence is presented, from ultra-violet absorption and oxidation studies, for considering the peltatins to be structurally related to podophyllotoxin.

THE ACTION OF SUBSTANCES EXTRACTED FROM PODOPHYLLIN ON SARCOMA 37 IN MICE. J. LEITER, V. DOWNING (by invitation), J. L. HARTWELL (by invitation), AND M. J. SHEAR. (National Cancer Institute, Bethesda 14, Md.)

A single subcutaneous injection of 10 to 50 micrograms per gram of body weight of the crude drug podophyllin produced softening, hemorrhage, and necrosis in sarcoma 37. The microscopic effects, previously described by MacCardle and Downing (1947) and 1948, were marked pyknosis of the tumor cells as well as arrest in mitosis. Similar effects were noted with a single injection of 3 crystalline components isolated from the crude drug, *viz.*, podophyllotoxin, *alpha*- and *beta*-peltatin. Quercetin, another known component in podophyllin, produced no such effects at doses up to 1000 micrograms per gram. Picropodophyllin and the sodium salt of podophyllinic acid produced no visible gross effects and no pyknosis at doses up to 500 and 2000 micrograms per gram respectively, although both produced distorted mitotic figures.

The minimum effective dose (MED) of podophyllotoxin, *alpha*- and *beta*-peltatin ranged between 2 and 5 micrograms per gram, depending on the vehicle employed. The maximum tolerated dose (MTD) of the three chemicals ranged from 25 to 60 micrograms per gram. The ratio of MTD to MED in mice for a single injection of podophyllotoxin, *alpha*- and *beta*-peltatin was about 10, 20, and 30, respectively.

Different routes of administration, *viz.*, subcutaneous, intravenous, and oral, all produced similar effects. A considerably higher dose level was required for the oral route (about 50 micrograms/gram), and the margin between the MTD and MED was much narrower by this than by the other routes.

Alpha- and *beta*-peltatin were soluble in aqueous solutions containing one to two equivalents of sodium hydroxide but had little, if any, solubility in olive oil. Podophyllotoxin was soluble (>5 per cent) in propylene glycol, moderately soluble (0.5 to 1.0 per cent) in olive oil, but only slightly (about 0.01 per cent) in water. All three were active against tumors in all the vehicles listed.

ACTION OF PELTATINS ON LYMPHOMAS AND OTHER TUMORS IN MICE. EZRA M. GREENSPAN (by invitation), J. LEITER, AND M. J. SHEAR. (Chemotherapy Section, National Cancer Institute, Bethesda 14, Md.)

The effect of subcutaneous injection of alkaline aqueous solutions of *alpha*- and *beta*-peltatin was studied on a variety of intramuscularly transplanted tumors. These included: an acute stem-cell leukemia, a metastasizing lymphosarcoma, a local lymphosarcoma, an adenocarcinoma, and a melanoma, all in mice; and a polymorphic rat carcinoma. A single subcutaneous injection regularly induced extensive gross and microscopic damage in the lymphoid tumors at low dose levels. Maximum tolerated doses were necessary for induction of consistent and extensive cellular damage in the adenocarcinoma, melanoma, and rat carcinoma.

Repeated injection of *alpha*-peltatin in mice bearing lymphoid tumors produced shrinkage of tumors and of metastases, retardation of tumor growth, shrinkage of spleen, and increased survival. *Alpha*-peltatin produced a leucopenia and a delay in infiltration of blast cells in blood and bone marrow of leukemia-bearing mice. Com-

plete regressions were not observed. Multiple low doses were well tolerated in mice bearing established palpable tumors. A large ratio of MTD/MED for a single subcutaneous injection was noted in animals bearing lymphoid tumors. The ratio for both peltatins was in the same range.

Mortality and weight loss, in animals bearing lymphoid tumors, were found to depend on the age and size of the tumors and on the extent of induced tumor damage as well as on the amount of drug administered. Shrinkage and histopathological changes were noted in the thymus, spleen, other lymphoid tissues and testis at maximum tolerated doses.

EFFECT OF A SINGLE INJECTION OF COLCHICINE, COLCHICINE DERIVATIVES AND RELATED COMPOUNDS ON MOUSE TUMORS. V. DOWNING (by invitation), J. L. HARTWELL (by invitation), J. LEITER, and M. J. SHEAR. (Chemotherapy Section, National Cancer Institute, Bethesda 14, Md.)

Colchicine and ten derivatives, each at its maximum tolerated dose, were injected subcutaneously into mice bearing week-old implants of sarcoma 37. Eight of these derivatives (colchicine, *N*-benzoyltrimethyl colchicinic acid methyl ether, trimethyl colchicinic acid methyl ether *d*-tartrate, *N*-acetylcolchinol and its methyl ether, *N*-acetylcolchinol and its methyl ether, and colchinol) all produced effects similar to colchicine, *viz.*, grossly visible hemorrhage and necrosis, marked mitotic arrests, distorted mitotic figures, and induced generalized pycnosis. (MacCardle and Downing, Cancer Research, 7:717, 1947; MacCardle, *ibid.*, 8, 1948. In press.) *N*-benzoyltrimethylcolchicinic acid produced some mitotic arrests but no pycnosis, whereas trimethylcolchicinic acid was entirely negative grossly and microscopically at doses up to 400 micrograms/gram body weight. The MTD of these derivatives was 10 to 450 times that of colchicine; trimethylcolchicinic acid methyl ether *d*-tartrate gave the widest range between the MTD (100 micrograms/gm. body weight) and the minimum effective dose (MED), namely, 2 micrograms/gram of body weight. Seven derivatives were soluble in an aqueous vehicle; 3 were injected as a suspension in oil. The active compounds also damaged other types of mouse tumors, both transplanted and chemically induced.

Approximately 18 phenanthrene derivatives yielded negative results in the same screening procedure, in which the mice were sacrificed at 8, 24, and 48 hours after injection of the compound. Of 163 diphenylethylamines similarly examined, 64 induced damage in sarcoma 37. Microscopically, however, this damage appeared to be different from the effects produced by the colchicine type of compound.

PROPERTIES OF THE PRODUCTS OF HYDROLYSIS OF SERRATIA MARCESCENS POLYSACCHARIDES. HUGH J. CREECH, MARTHA W. WHARTON (by invitation), REED F. HANKWITZ, JR. (by invitation), D. R. A. WHARTON (by invitation), and IRENE C. DILLER. (Lankenau Hospital Research Institute and The Institute for Cancer Research, Philadelphia 30, Pa.)

Since it is important clinically to reduce the toxicity and antigenicity of the tumor-necrotizing polysaccharides from *Serratia marcescens*, exploration has been made of the action of glycophosphatases and other methods of hydrolysis on these polysaccharide-lipid complexes. Preparations of enzymes, obtained by processing rat intestine, were allowed to act upon the complexes at pH 5 and 9. The enzymatic hydrolyses were conducted within a cellophane sac surrounded by suitable buffer solutions which were changed frequently. Tests of the antigenicity, toxicity and tumor-necrotizing activity were made on the total non-dialyzable product at several stages during the hydrolysis. The extent of hydrolysis was determined by suitable micro methods of analysis for glucose and phosphorus.

With an increased degree of hydrolysis, there was a corresponding decrease in the ability of the product to elicit the formation of agglutinins in mice. The products obtained by hydrolysis at pH 5 were more toxic than those obtained at pH 9; a single injection of the products obtained by hydrolysis at pH 5 conferred a greater tolerance on mice toward a subsequently administered lethal injection of unaltered polysaccharide than those obtained under alkaline conditions. Variations occurred among the products obtained at each pH and these could not always be correlated with the degree of hydrolysis. A lack of parallelism was also noted between the extents of tumor-necrotization and hydrolysis. It is anticipated that fractionation of the non-dialyzable products will provide interesting materials for study.

STUDIES ON THE INHIBITION OF SARCOMA 180 IN MICE. C. CHESTER STOCK, KANEMATSU SUGIURA, ALICE E. MOORE (by invitation), and C. P. RHOADS. (Division of Experimental Chemotherapy, Sloan-Kettering Institute for Cancer Research, New York, N.Y.)

Inhibition of the development of Sarcoma 180 in mice has been developed as a method of screening materials for adverse effects upon tumor tissue. Twenty-four hours after subcutaneous implantation of Sarcoma 180 into the axillary region of mice, intraperitoneal injections of the chemotherapeutic agent in maximum tolerated doses are initiated and continued twice a day for 7 days. At the end of the injection period the tumors are measured in two diameters by calipers. The inhibition of the tumors in the treated animals is based on the development of the tumors in relation to the untreated controls.

In a study of over 800 compounds the test has eliminated approximately 90 per cent of the materials from further study; yet, it appears sufficiently sensitive to suggest new types of compounds worthy of exploration. The screening test is merely a preliminary to additional studies of therapeutic and cytological effects on Sarcoma 180 and for studies on a spectrum of mouse and rat tumors.

The results obtained with miscellaneous compounds including carbamates, fluorenes, nitrogen mustards, anti-biotics, folic acid analogs, and related compounds are summarized.

EFFECTS OF PODOPHYLLOTOXIN AND PICROPODOPHYLLIN ON BLOOD CELLS AND OTHER TISSUES OF RATS AND MICE. MARGARET G. KELLY (by invitation), ROSS C. MACCARDLE, and PAUL K. SMITH. (Department of Pharmacology, George Washington University Medical School, Washington, D.C., National Cancer Institute, Bethesda, Md., and Department of Pharmacology, George Washington University Medical School, Washington, D.C.)

It was reported last year by this and other laboratories that administration to rats of podophyllin and some podophyllin derivatives produced a transitory leukopenia. The possibility that this effect was mediated through the adrenal glands was investigated by following the changes in the eosinophiles and by histologic study of the adrenal glands.

Counts were made at one and twenty-four hours after single intraperitoneal injection of 1 and 10 mg. per kilo of podophyllotoxin and 7 and 75 mg. per kilo of picropodophyllin. A significant decrease in the number of eosinophiles in the circulating peripheral blood was found and was accompanied by depletion of the osmophilic granules in the adrenal glands.

Swiss mice bearing 6-day old intramuscular implants of Sarcoma 37 in the right thigh received a single intraperitoneal injection of either podophyllotoxin (10 mg. per kilo) or picropodophyllin (75 mg. per kilo). The mice were killed at intervals ranging from 8 hours to 10 days after injection and no significant change was observed in histologic preparations of liver, spleen, lung, kidney, and intestine. The tumor tissue, however, showed necrosis and mitotic arrests and the osmophilic granules were greatly depleted in the fasciculae of the adrenal gland. Chronic toxicity changes in tissues are being studied in normal Swiss mice.

GLYCOLYTIC INHIBITOR THERAPY IN HUMAN MALIGNANT NEOPLASIA. MAURICE M. BLACK, ISRAEL S. KLEINER (by invitation), and HERMAN BOLKER (by invitation). (Department of Biochemistry, New York Medical College, New York, N.Y.)

In vitro metabolic studies of tumor tissue reveal a striking uniformity among diverse tumor types in regard to accentuation of aerobic and anaerobic glycolysis. The use of the glycolytic inhibitors fluoride, iodoacetate, and malonate on a series of more than 100 cases of diverse forms of human cancer was accompanied by objective evidence of tumor inhibition in cases of acute leukemia, Hodgkin's Disease and lymphosarcoma. This was not the case in patients with carcinoma of the colon, fundus uteri, ovary, pancreas, rectum, or in chronic leukemia, melanoma, chorioepithelioma testis, or with squamous cell cancers of the cheek and pharynx. Partial or questionable therapeutic response occurred in cancers of the breast, adrenal cortex, cervix, lung, stomach, testes, and in fibrosarcoma. These observations suggest that while certain cancer types behave as if they were particularly dependent on glycolytic mechanisms for their energy requirements, this is certainly not the case for all or even a majority of them.

The limitation of the therapeutic efficacy of these agents to individual tumors or related neoplastic types may be explained by either of two possible alternatives. Either the inhibitory effect with these compounds represents an action on a unique metabolic or structural feature of these growths, or the concept of an essential entity of all malignant neoplasia as tacitly predicated on the basis of chemical and cytological observations is false or inadequate. The *in vitro* biochemical characterization of tumor tissue may be a measure of a manifestation of malignancy rather than of its subtle essence or it may fail to reflect the ability of tissues to adapt to an unfavorable environment by alterations of their metabolic pathways.

A STUDY OF N-IODOACETYL AMINO ACIDS IN RELATION TO INHIBITION OF TUMOR GROWTH. ORRIE M. FRIEDMAN and ALEXANDER M. RUTENBURG. (Introduced by A. Seligman.) (Department of Chemistry, Harvard University, Cambridge, Kirstein Laboratory of Surgical Research, Beth Israel Hospital, Boston, and Department of Surgery, Harvard Medical School, Boston, Mass.)

Since toxic substances related to essential metabolites seemed of interest for a study of inhibition of growth of tumors, derivatives of amino acids were prepared which were toxic and which could be readily labelled with a radioactive isotope. N-iodoacetyl derivatives of tryptophane, leucine and phenylalanine have been prepared. The relative toxicity in Swiss mice of these three substances and iodoacetamide was determined and the ability of the four substances to inhibit the growth of Sarcoma 37 in this test animal was studied. The results have indicated that these substances inhibit the growth of this tumor significantly, to different extents and in a manner apparently unrelated to systemic toxicity.

The radioactive analogues of the three iodoacetyl amino acids and iodoacetamide have been prepared by the use of I.¹³¹. The concentration of radio-activity and its disappearance from blood, tumor and liver following intravenous injection of these substances have been determined. Radioactivity has been found in the three tissues in significant amounts, the concentration of activity in tumor being consistently greater than in liver and less than in blood. The rate of disappearance of radioactivity from tumor in the case of the three amino acid derivatives followed a similar characteristic pattern different from that of iodoacetamide.

INTRACYTOPLASMIC PHYSIOLOGICAL MEDIA. M. J. KOPAC. (Department of Biology, Washington Square College of Arts and Science, New York University, New York 3, N.Y.)

Cell inclusions and submicroscopic particulates isolated by centrifugal fractionation especially for enzymic determinations require media that maintain such cytoplasmic fractions in essentially native states. Such media are being developed and evaluated by methods involving microinjection and micro-surface chemical techniques. Experimental media that produce no appreciable

disturbances in the cytoplasm on microinjection, nor changes in structure and properties of mitochondria, microsomes, and nuclei, streaming, or sol-gel transformations, should preserve formed components, on disintegration of the cell, in reasonably native states for subsequent isolation.

Thus far, the best medium for Myxomyete and ameba cytoplasm contains KCl, CaCl₂, and NaCl, in which K/Na = 9, and (K + Na)/Ca = 40. A satisfactory medium for isolating *Arbacia* egg inclusions contains KCl and Na-citrate since ionic Ca must be avoided. For micromanipulative studies on salivary gland chromosmes, the medium must be K-rich and Ca-free.

Previous work has shown that cytoplasmic proteins in intact cytoplasm do not become surface denatured at oil/water interfaces. Accordingly, intraeytoplasmic media were tested by surface chemical methods that can measure changes in surface denaturation of proteins at oil/water interfaces established in living cells. By simultaneously injecting aqueous media with the indicator oil, the action of these media can be compared with that produced on introduction of the oil/water interface alone (traumatic cytolytic effect).

Preliminary measurements indicate that media otherwise satisfactory for ameba cytoplasm significantly increase the surface denaturation of cytoplasmic proteins of the ameba. Intraeytoplasmic media, therefore, should be further modified by adding calcium binders, chelating agents, nucleic acids, or anti-surface denaturating agents exemplified by *bis*-amidinomethylbenzyl or protamine.

K-rich, Na-poor solutions fortified with either nucleic acids or *bis*-amidinomethylbenzyl produce much less surface denaturation of cytoplasmic proteins. None, however, produces the low degree of surface denaturation obtained at oil/water interfaces established without previous injection of intraeytoplasmic media. Continued investigations should yield better media for isolating complete enzymic systems in the form, probably, of submicroscopic cytoplasmic particulates.

POLAROGRAPHIC STUDIES ON THE LIPIDS OF EPIDERMIS UNDERGOING CARCINOGENESIS WITH METHYLCHOLANTHRENE. C. CARRUTHERS AND V. SUNTZEFF. (Department of Anatomy, Division of Cancer Research, Washington University Medical School, St. Louis, Mo.)

An alteration in a lipid occurs during the process of epidermal carcinogenesis in mice. Polarography of the lipid extractable material of mouse epidermis undergoing carcinogenesis was carried out in unbuffered and buffered mixtures of water, dioxane (50 per cent by volume) and tetrabutylammonium iodide as supporting electrolyte. The lipids of normal and hyperplastic epidermis gave a double or two polarographic waves while induced or transplantable squamous cell carcinomas showed only a single wave. In buffered solutions the waves of the lipids of normal and hyperplastic epidermis were found to be pH dependent. The single wave of the lipids of the carcinoma disappeared in buffered solutions showing that its presence in the unbuffered mixture was due to some lipid substance which acted as a buffer.

The lipid material of the carcinomas did have a very small amount of a reducible lipid, the half-wave potential of which was significantly more positive than that of the first wave of epidermis, and is probably a compound characteristic of the carcinoma. This information indicates that epidermis in becoming carcinomatous either loses the lipid having the double polarographic wave or that this lipid is qualitatively altered from that found in the carcinomas. Normal human epidermis has the same double polarographic wave as the mouse, and the process of carcinogenesis in both species seems to be similar with respect to these reducible lipids. Efforts are now in progress to ascertain the nature of the reducible lipids.

HISTOCHEMICAL STUDIES OF MOUSE HEPATOMAS PRODUCED BY CARBON TETRA-CHLORIDE. C. S. LEE (by invitation), R. E. STOWELL, AND A. VILLISANA (by invitation). (Departments of Oncology and Pathology, University of Kansas Medical School, and Department of Pathology, Washington University School of Medicine, St. Louis, Mo.)

The histochemical and cytological changes in the livers of mice following single and repeated feedings with carbon tetrachloride were followed through the stages of cirrhosis and hepatoma formation. At monthly intervals, 53 experimental and 15 control animals were started on the diet and all animals were killed and tissues fixed at one time. In addition to the usual trabecular hepatomas, growth with acinous and lymphangiomatous nature were seen.

Tissues were stained for alkaline phosphatase, glycogen, lipase, ribose nucleic acid, deoxyribose nucleic acid, lipids, bile ducts, and mitochondria. As compared with normal liver tissue cirrhotic liver showed only slight changes in alkaline phosphatase, glycogen and lipase reaction, while most of the hepatoma cells showed stronger staining reactions for alkaline phosphatase, glycogen, and reduced lipase reaction. Ribose nucleic acid was increased in some hepatomas. The differences in the histochemical staining reactions between the cirrhotic and hepatoma tissue were frequently pronounced.

After a single feeding of carbon tetrachloride the initial necrosis was followed by increased mitotic activity and regeneration. Alkaline phosphatase and glycogen decreased markedly the first day and then gradually increased especially in the central parts of the lobule. Lipase activity decreased through the third day and started to increase five days after treatment.

CYTICHEMICAL STUDIES OF NUCLEOPROTEINS IN NUCLEI OF A TRANSPLANTED TUMOR (SARCOMA 180). CECILIE LEUCHTENBERGER. (Department of Zoology, Columbia University, New York, N.Y.)

The nucleoprotein content of resting nuclei of a viable tumor tissue was compared with that of pyknotic nuclei of necrotic areas of the same tumor. The relative amounts of deoxyribonucleic acid (from the Feulgen reaction) and protein (from the Millon reaction) in the

different nuclear types were estimated in fixed sections by the microscopic photometric method of Pollister and Ris. The methylgreen staining was used to detect a change in the physical state of the nucleic acid. Cytologically 4 different types of tumor cells were selected for this study: a viable tumor cell (characterized by a distinctly basophilic cytoplasm, a spherical nucleus, and a large nucleolus) and three progressive stages of pyknosis as judged by the increasing amount of condensation of the chromatin and reduction of the nuclear volume. The earliest pyknotic stage is characterized by a rounding up of the nuclei, by a marked reduction of the nuclear volume, by a decrease in the protein content, and a considerable reduction of the affinity of the chromatin for methylgreen. The total desoxyribonucleic acid, as indicated by the Feulgen stain, remains practically unchanged in comparison with the nuclei of the viable tumor cells. The loss of the stainability with methylgreen suggests that depolymerisation of the desoxyribonucleic acid has taken place. Later pyknotic stages show a further decrease in the nuclear volume and protein content and a partial loss of the desoxyribose nucleic acid. Whereas, in pyknosis, the reduction in the nuclear volume and protein content and the nucleic acid depolymerisation are extensive at an early stage, the actual decrease in the amount of desoxyribonucleic acid does not become pronounced until much later.

PHASE MICROSCOPE STUDIES OF MALIGNANT CELLS. ROBERT P. ZANES, JR. (by invitation), DOROTHY ESHBAUGH (by invitation), and HERMAN A. HOSTER. (Ohio State University College of Medicine, Columbus, Ohio)

Scraping of the cervix and aspiration from the vaginal canal and cervix at the time of pelvic examination have been used extensively in attempting to establish or rule out the presence of neoplastic disease using the Papanicolaou technique. This report is a preliminary discussion of observations made on material obtained from the same source and studied by means of the phase contrast microscope using unstained and supravitally stained smears. The specimens were studied within two hours after preparation in order to confine observation to cells in the living state. An attempt has been made to correlate these observations with the clinical findings, the histo-pathologic pattern, Papanicolaou smear interpretations, and changes due to recent radiation therapy.

In two hundred cases of normal individuals and individuals with biopsy-proven neoplasia, the cyt-morphologic criteria suggesting malignancy appeared to be: (a) an increase in nuclear size and irregularities in nuclear shape; (b) the presence of multiple, assymetrical nuclei; (c) the presence of large, irregular and multiple nucleoli; and (d) the presence of refractile cytoplasmic bodies, sometimes identical in size and shape and occasionally varying markedly. These bodies are observed arranged in a ring around the nucleus or in a rosette formation in a host of the nucleus.

Preliminary comparisons with the results of the Papanicolaou examination reveal agreement in approximately 70 per cent of the cases studied. No statement can be made at this time concerning an increased effi-

ciency of the phase contrast smear technique as compared with that of the Papanicolaou technique. The phase technique has the advantage of providing supplementary diagnostic information at the time of the pelvic examination without the delay associated with fixation and staining.

STUDIES WITH A RADIOACTIVE IODOTETRAZOLIUM COMPOUND AND WITH A NEW TETRAZOLIUM SALT WHICH YIELDS A BLUE PIGMENT ON REDUCTION. ARNOLD M. SELIGMAN, RAPHAEL GOFSTEIN (by invitation), and ALEXANDER M. RUTENBURG (by invitation). (Department of Surgery, Beth Israel Hospital, Boston, and Harvard Medical School, Boston, Mass.)

Diphenyl (*p*-iodophenyl) tetrazolium chloride has been prepared with radioactive iodine (I^{131}) in a series of synthetic steps from 1 mg. of aniline and benzal phenyl hydrazone in a single reaction vessel. Following intravenous injection of the radioactive tetrazolium salt into tumor bearing mice, radioactivity in the circulating blood dropped in $\frac{1}{2}$ hour to 2 to 4 per cent of that injected. The highest concentrations of radioactivity were found in kidney, liver, and lung. Radioactivity persisted at higher levels in lung and in mesenteric fat. Sarcoma 37 contained less radioactivity than most other tissues.

A water soluble, pale yellow tetrazolium salt has been prepared in good yield from the formazan obtained from benzal phenyl hydrazone and diazotized dianisidine. The formazan produced by enzymatic or chemical reduction of the tetrazolium compound is a deep blue water insoluble pigment. This pigment when dissolved in organic solvents may be measured colorimetrically in concentrations less than 0.5 microgram per cc. The tetrazolium compound is about as toxic as triphenyl tetrazolium chloride. It is being used in the development of histochemical methods for demonstrating oxidation-reduction systems in normal and neoplastic tissues.

NEW METHODS FOR THE HISTOCHEMICAL DEMONSTRATION OF INTRACELLULAR ENZYMEs. ARNOLD M. SELIGMAN, and LEON H. MANHEIMER, and MARVIN NACHLAS (by invitation). (Department of Surgery, Beth Israel Hospital, Boston, and Harvard Medical School, Boston, Mass.)

Utilizing the method first developed by Menten *et al.* for alkaline phosphatase, substrates have been prepared from naphthols which are split by intracellular enzymes in the presence of stabilized diazonium salts. Immediate coupling, which results with the naphthols as they are released, produces insoluble colored pigments in tissue sections at the site of the enzymatic activity. The following enzymes can be demonstrated: alkaline phosphatase, three varieties of acid phosphatase, non-specific esterase, lipase, sulfatase, and *B*-blneuronidase.

COLOR REACTIONS WITH MALIGNANT SERA. EMIL WEISS. (Department of Pathology, Peoples Hospital Chicago 16, Ill.)

A number of aniline dyes in dilutions 1:10,000 do not show any color changes on addition of malignant sera. Sera of normal individuals, those afflicted with various diseases or benign tumors turn the original blue color of the same dyes green. The glassware must be clean and free of any acid or dye. The sera must be fresh and thoroughly centrifuged. Turbid, hemolyzed, or icteric sera can not be used. The dyes are kept in 0.1 per cent stock solutions of rubbing alcohol (70 per cent isopropyl alcohol). The stock solution is diluted with distilled water 1:10 before use. To 0.5 cc. of the diluted stock solution of the dye 0.5 cc. of serum is added, the tube is shaken for a few seconds and the color changes are noted. A positive control containing a known malignant serum and a negative control containing a known normal serum are handled in the same manner. A dye control correspondingly diluted with water also is used. The tubes are placed overnight or for 12 hours in the refrigerator and then the final reading is made. Eventual color changes become more distinct after standing several hours. Over 300 sera were examined with the above technic. Positive (blue) reactions were obtained in 89.1 per cent of malignant sera. Azur II, toluidine blue, trypan blue, and victoria blue were found to be equally useful. This method applies to all types of malignancy. Hodgkin's disease and various forms of leucemia give consistently positive results.

FURTHER STUDIES ON THE RELATIONSHIP OF MUCOLYTIC ENZYMES TO THE INVASIVE GROWTH OF CARCINOMAS AND SARCOMAS IN MICE. WILLIAM L. SIMPSON, A. R. T. DENUES (by invitation), AND PATRICIA J. KELLER (by invitation). (Detroit Institute of Cancer Research, Detroit 1, Mich.)

The capacity for invasive growth forms the most critical point of differentiation of malignant cells from normal cells. In previous studies by Simpson *et al.*, enhancement of this property has been associated with injections of the spreading factor from testes, hyaluronidase. Coman and his collaborators failed to find such an effect with a benign tumor and with a transplantable sarcoma. Additional series of mouse transplantable squamous cell carcinomas and fibrosarcomas have been analyzed for the effects of local injections of hyaluronidase on invasive growth and metastasis. The findings in these experiments will be presented in detail.

SEVERAL FACTORS THAT INFLUENCE *p*-DIMETHYLAMINOAZOBENZENE CARCINOGENESIS IN THE RAT. PAUL N. HARRIS. (Lilly Research Laboratories, Eli Lilly and Co., Indianapolis, Ind.)

Purified diets containing .09 per cent of *p*-dimethylaminoazobenzene, were administered continuously in these experiments until the rats died or were killed for microscopic examination of their tumors. The rapidity of tumor development was influenced by the strain of rat employed. There was no difference between the Evans, Sprague-Dawley, and Harlan strains (the Harlan strain, obtained from a local breeder, is descended

from the Wistar strain). As compared with these strains, tumor development in rats obtained from the Wistar Institute was somewhat retarded, and in the Carworth Farms strain was even more delayed. Variation in the amount of cottonseed oil (5, 10, and 20 per cent) in the diet had no effect upon carcinogenesis. With a diet containing 20 per cent of olive oil, tumor development was slower than with diets containing 5 per cent and 10 per cent. A diet containing 5 per cent of corn oil gave less rapid tumor development than diets containing 10 per cent and 20 per cent. With a low level of riboflavin (2 mg./kg.), there was no difference in tumor development in rats given 10 per cent and 30 per cent casein diets, but with a riboflavin level of 100 mg./kg., there was great retardation of carcinogenesis with a 10 per cent casein diet. With a constant level of riboflavin (7.9 mg./kg.) and pyridoxine (3 mg./kg.), a diet containing 10 per cent of casein gave less rapid tumor development than did one containing 7 per cent of casein and 3 per cent of liver extract.

ENZYME INHIBITION IN RELATION TO CHEMOTHERAPY. W. W. ACKERMANN (by invitation), and V. R. POTTER. (McArdle Memorial Laboratory, University of Wisconsin, Madison 6, Wis.)

Studies are presented which emphasize the importance of the dissociation constant of the enzyme-inhibitor complex in relation to enzyme concentration. When the constant is sufficiently small the per cent inhibition of the enzyme will be determined not only by the concentration of the inhibitor but also by the concentration of the enzyme. By means of a graphic method, in which the rate of reaction was plotted against enzyme concentration, it was possible to show that in the case of certain inhibitors having very small dissociation constants the activity of the enzyme was approximately proportional to $(E) \cdot (EI)$, where (E) was the total concentration of enzyme and (EI) was approximately the concentration of added inhibitor. To describe the relationship a general equation was developed to express the concentration of the enzyme-substrate complex in terms of the total amount of enzyme, the amounts of substrate and inhibitor and the dissociation constants of the enzyme substrate and enzyme-inhibitor complex.

From the standpoint of chemotherapy, these studies lead to the suggestion that it may be possible to inactivate specifically an enzyme in cancer tissue even though the enzyme is not unique to cancer tissue. If a highly specific inhibitor with a low dissociation constant is used, and if the enzyme is present in lower concentrations in cancer tissue than in normal tissues, it should be possible to inactivate all of the enzyme in the cancer while inactivating smaller percentages of the enzyme in normal tissues.

PRODUCTION OF PROFOUND CHANGES IN BACTERIA BY RHYTHMIC EXPOSURE TO HEAT. R. R. SPENCER and M. B. MELROY. (National Cancer Institute, Bethesda, Md.)

Heat has been shown to be both a carcinogen and mutagen (Demerec. Brit. J. Cancer, 2: 114-117, 1948).

By the use of high temperatures, a technique has been developed which results in profound permanent alteration of bacteria. The fundamental principle underlying this technique is a rhythmic injury-repair cycle of exposure extending over a long period of active multiplication of the species propagated in serial cultures.

Analysis of the experimental data reveals the importance of three rhythmic factors in the adaptation of bacterial species to an unfavorable environment (high temperatures). These are: a) *The temperature rhythm* (the duration of the interval of exposure); b) *The rest rhythm* (the duration of the period of freedom from heat); c) *The transfer rhythm* (the interval of transfer of the serial cultures).

These rhythms and their interrelationships determine to a large extent the survival and adaptation of the species or its failure to survive. It is suggested that carcinogenesis may be basically a problem in survival and adaptation of somatic cells to unfavorable environments over long periods of time.

A HISTOPATHOLOGIC AND GENETIC STUDY OF TUMORS OF THE FORESTOMACH IN MICE TREATED WITH A SINGLE SUBCUTANEOUS INJECTION OF METHYLCHOLANTHRENE. M. A. BAGSHAW (by invitation), and L. C. STRONG. (Department of Anatomy, Yale University School of Medicine, New Haven, Conn.)

Approximately 3,000 mice of the pBr subline were injected subcutaneously with 1.0 mg. of methyleholanthrene at 60 days of age. Local lesions developing at the site of injection were previously described. An analysis of the first 78 tumors of the forestomach is presented. The mean latent period was 454 days.

Hematoxylin-eosin, Laidlaw's silver reticulum, Masson, and Van Gieson's stains were employed. Ninety-nine per cent of the tumors were classified as either differentiated epidermoid carcinomas, spindle cell neoplasms, or mixed tumors. One adenocarcinoma and one round cell tumor were found. The differentiated epidermoid carcinomas were composed of sheets of epithelial cells containing basal cells, spinous cells, and keratinized elements. The spindle cell neoplasms were characterized by bands and whorls of spindle shaped cells which contained scanty, ill defined cytoplasm and long, oval, usually hyperchromatic nuclei. The mixed tumors contained epidermoid as well as spindle cells. Comparison of the above three types revealed numerous characteristics in common. The term, sarcoma, for the spindle cell lesion seemed unjustified. Metastases throughout the abdomen and thorax occurred frequently. Transplantation of the epidermoid carcinoma to homologous hosts was successful. Intraperitoneal transplants resulted in tumors which killed the new hosts within 2 or 3 weeks and were morphologically identical to the spindle cell neoplasms.

Genetic analysis revealed relatively more stomach tumors, irrespective of type, in a single lineage over a period of 5 generations.

RELATIONSHIP BETWEEN THE HAIRLESS GENE AND SUSCEPTIBILITY TO INDUCED PULMONARY TUMORS IN MICE. W. E. HESTON and MARGARET K. DERINGER. (National Cancer Institute, Bethesda, Md.)

High-pulmonary-tumor strain A mice were outcrossed to a strain of pink-eyed hairless mice and the F₁ progeny in turn backcrossed to the pink-eyed hairless strain. This backcross generation provided a test for linkage between susceptibility to pulmonary tumors and both the pink-eye gene (*p*) and the hairless gene (*hr*). Ninety-two of these backcross mice were injected intravenously each with 0.5 mg. dba dispersed in 0.5 cc. horse serum at from 2 to 3 months of age and were killed 5 months later and the number of pulmonary tumors in each was recorded. No linkage between susceptibility to pulmonary tumors and the pink-eye gene was evident since the proportion of pink-eyed segregants with pulmonary tumors and the mean number of nodules found in the pink-eyed segregants was not significantly different from that of the non-pink-eyed segregants. There was, however, a relationship between susceptibility to the induced pulmonary tumors and the hairless locus. The hairless segregants were less susceptible. When the hairless mice were compared with haired in regard to proportion with tumors, the difference was borderline in significance but when compared in regard to average number of nodules per individual, a more delicate measure of susceptibility, the difference was highly significant statistically. Growth curves of non-injected backcross mice indicated no difference between pink-eyed and non-pink-eyed segregants, but the hairless segregants were smaller than the haired. Possible relationship between the effect of the hairless gene on growth and its effect on susceptibility to pulmonary tumors is discussed.

A "MATERNAL INFLUENCE" ON THE GROWTH OF A TRANSPLANTABLE TUMOR IN MICE. MORRIS K. BARRETT and WALTER C. MORGAN (by invitation). (National Cancer Institute, Bethesda 14, Md.)

That the incidence of spontaneous tumors and the successful inoculation of transplantable tumors are strongly influenced by genetic background has been long established. It is also known that there is a "maternal influence" on the incidence of certain spontaneous tumors and the "take" of certain transplantable tumors.

A transplantable mammary carcinoma which originated in a C3H mouse was inoculated into F₁ hybrid mice derived from reciprocal crosses between strain C3H and strain C (B alb C). The tumor grew progressively in all mice but a difference in the rate of growth was observed that depended upon which strain was the maternal line of the hybrids and which the paternal. In all of eleven experiments the average weight attained in three weeks by the tumors was greater in the case of tumors growing in hybrids of the C3H maternal line. Under the conditions of these experiments the maternal influence noted was independent of the sex and age of the hosts, the absolute size of the tumors, the presence of

intercurrent disease, the donor of the graft (whether inbred or hybrid) and annual variations in laboratory conditions. The nature of the factor or factors which account for this maternal influence was not disclosed by these experiments.

THE EFFECT OF CROWDING ON THE PENETRANCE OF AN HEREDITARY MELANOMA OF DROSOPHILA MELANOGASTER.* M. H. HARNLY (by invitation), E. D. GOLDSMITH, and F. FRIEDMAN (by invitation). (Washington Square College and the College of Dentistry, New York University, New York, N.Y.)

Crowding has been found to affect the penetrance of a benign hereditary melanoma in *Drosophila melanogaster*. Single pair matings in 1×4 inch vials gave a significant difference in penetrance of the tumor for one and eight day egg laying periods. To determine the effect of known intensity of crowding (developing animals per vial) on the percentage of adult animals having this melanotic tumor, groups varying from 5 to 200 just hatched larvae were placed in vials of food. Approximately 85 per cent of the adults had tumors at all concentrations and 400 larvae per vial has given a similar value. When the longer egg laying period was simulated by adding 100 larvae per day for four days in the vials (a total of 400 larvae per vial) the penetrance dropped from 85 per cent to 54 per cent on the large number of animals tested. Obviously, crowding as such does not affect the penetrance of this hereditary melanoma. The data suggest that the critical time in development for the onset of tumor growth is during the first larval instar and environmental changes resulting from crowding determine in large part the penetrance or non-penetrance of this melanoma. These points are now under investigation.

ADRENAL CORTICAL TUMORS IN A RECIPROCAL CROSS. GEORGE W. WOOLLEY and MARGARET M. DICKIE (by invitation). (Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine)

A cross was made reciprocally between two strains of mice to see if maternal influences were of importance in the inheritance of adrenal cortical tumors. The results indicate that there is no major maternal influence, such as has been found with mammary tumors, associated with the inheritance. The tumors were of the type obtained following gonadectomy. Some observations on tumor development are included.

AGE AND LYMPHOMA INCIDENCE IN CF-1 MICE. AUSTIN M. BRUES, MIRIAM P. FINKEL, HERMANN LISCO, and GEORGE A. SACHER (by invitation). (Argonne National Laboratory, Chicago, Ill.)

Three large groups of CF-1 female mice, a total of 2500 animals, have been maintained throughout life as controls for radiation experiments. The median life span of these animals is about 600 days and about 1 per cent survive beyond 850 days.

* Aided by a grant from The National Cancer Institute, National Institute of Health, U.S. Public Health Service.

Throughout most of life, the susceptibility to lymphoma and lymphatic leukemia is approximately doubled at intervals of 85 days. After 700 days of age no further increase in morbidity rate is observed. This pattern of incidence is similar to that seen in the case of many human tumors. The three groups of mice were obtained over a three-year period, and the age incidence of lymphoma has remained nearly constant.

MONOZYGOTIC TWINS WITH SIMILAR BREAST PATHOLOGY SUGGESTING THE ORIGIN OF MALIGNANT FROM BENIGN NEOPLASIA. DUDLEY JACKSON, JR. (by invitation), DUDLEY JACKSON, SR. (by invitation), F. W. STEINBERG (by invitation), and C. P. OLIVER. (Nix Memorial Hospital, San Antonio, and University of Texas, Austin, Texas)

Twins "A" and "B" have strikingly similar breast pathology. Cysts were removed from "A's" right breast at age 28. When the twins were age 36, "A" had a bleeding left nipple, and a simple mastectomy was performed. "B" had cysts removed from both breasts and "A" had a cyst removed from her right breast in 1941. "A's" tissue showed "epithelial hyperplasia." "B" had another cyst excised a year later, and this also had "epithelial hyperplasia." "B" developed the next tumor July, 1945, and the pathologist reported "chronic cystic mastitis, multiple papillary cystadenomas and intracanalicular adenofibroma and cysts with marked papillary infoldings." In October, 1945, "A's" right breast showed epithelial hyperplasia and pathological features very similar to "B's" report of three months earlier. "B" had a lump in her left breast in October, but delayed surgery. Simple mastectomy was done on "A's" right breast in December at the request of the pathologist who considered the lesions found on the biopsy in October to be "premalignant." Twelve days later, biopsy of "B's" left breast showed intraductal adenocarcinoma and radical mastectomy was done. Histological examination of the tissue was suggestive of a transition from papillary epithelial hyperplasia to malignancy. The twins' monozygotic origin was determined from studies of the AB, MN, and blood types, PTC taste thresholds, dermatoglyphics, and other physical characteristics. The family history shows cancer, glandular deficiency and diabetes among the twins' relatives. The twins' parents and other relatives were short lived. The twins and some relatives are highly infertile.

COMPLEMENT FIXATION IN ANIMAL NEOPLASIA. II. DEVELOPMENT OF THE REACTION IN NEW ZEALAND RABBITS CARRYING THE BROWN-PEARCE CARCINOMA. LESTER D. ELLERBROOK, MARK RHEES, and HELEN THORNTON (by invitation), and STUART W. LIPPINCOTT. (Department of Pathology, School of Medicine, University of Washington and the Cancer Control Division, National Cancer Institute)

The inoculation of the neoplasm which had been carried in New Zealand white rabbits into adult animals of the same breed resulted in the production of definitely

positive reactions in the majority of the animals developing tumors. Tests of sera obtained by serial bleedings of these animals as a rule showed definite complement fixation at 2 to 3 weeks after inoculation and the degree of fixation tended to increase with the continued development of the neoplasm.

The maximum reactions were obtained with sera inactivated at approximately 60° C. Titers were calculated from the volumes of complement required to reach the end-point of 50 per cent hemolysis in the presence of normal serum and of test serum, both alone and in the presence of antigen. Thus far definitely positive reactions were not obtained with rabbits bled serially or with those inoculated with such materials as turpentine, human serum, or normal rabbit muscle.

NATURAL AND IMMUNE ANTIBODIES IN MICE WITH LOW AND HIGH TUMOR INCIDENCE. ISRAEL DAVIDSON (by invitation), and KURT STERN. (Mount Sinai Medical Research Foundation and the Chicago Medical School, Chicago, Ill.)

Natural heteroagglutinins for sheep red cells and for human red cells were investigated in six inbred mouse strains: C57 black, Bagg albino C, C3H, dba, Marsh-albino, and Akm. Striking differences were found in the ability of the serum of these strains to agglutinate sheep red cells. Natural antisheep agglutinins were absent in 40 to 60 per cent of the serum of mice of the C3H, dba, Marsh-Albino, Bagg albino C and Akm strains; the agglutinins present in these strains showed low titers (less than 16). In contrast, animals of the C57 black strain showed presence of antisheep agglutinins in more than 90 per cent, with titers above 16 in more than 50 per cent. No such strain differences were found as to presence and titers of natural agglutinins for human red cells.

Determinations of antisheep agglutinins and hemolysins, following intraperitoneal injection of sheep red cells into C57 black, dba, and C3H animals, revealed a higher antibody production in the C57 black mice than in the two other strains. Similar results were obtained in the three strains in regard to antibodies produced by injection of human red cells.

The significance of these findings is discussed in the light of previously reported differences in the storing ability of these strains. Both observations may be related to differences in the reticulo-endothelial activity of the various strains.

STRUCTURAL AND FUNCTIONAL CHARACTERISTICS OF SPONTANEOUS THYROID TUMORS IN A SWORDTAIL FISH SPECIES.* AUBREY GORBMAN (by invitation), and MYRON GORDON. (Barnard College, Columbia University and New York Aquarium, New York Zoological Society, New York, N.Y.)

Tumorous enlargement and metaplasia of the thyroid has been observed in a relatively high incidence in older specimens of both sexes of swordtail, *Xiphophorus*

montezumae. Thyroidal tumors occur only rarely under similar laboratory and dietary conditions in related species: *Xiphophorus pygmaeus*, *Platypoecilus maculatus*, and *Platypoecilus xiphidium*. They have not been observed in *X. hellerii*, *P. variatus*, or *P. couchianus*. The diet for all species consists of fresh liver, Pablo cereal, live tubificid worms, and dried ocean shrimp. In nature *X. montezumae*, *X. pygmaeus*, and *P. variatus* are found living together occasionally in the Rio Axtla, San Luis Potosi, Mexico. *X. montezumae*'s greater susceptibility may indicate a specific genetic difference.

The main mass of the tumors in *X. montezumae* varying from 35 to 50 millimeters in length is about 5 mm. long and 4 mm. dorsoventrally. Histologically the tumor appears to consist mostly of a microfollicular and afollicular mass, well vascularized, and with abundant stroma. At the edge of the growth a few larger colloid-containing follicles may be found. Follicles may be found within bones and muscle, and heavily invading the gills, obviously interfering with respiratory function.

Administration of tracer radioiodine to tumorous animals and subsequent radioautography reveals that the bulk of the tumor takes up no iodine. Almost normal iodine intake is exhibited by the few peripheral colloid-filled follicles.

THE EFFECT OF A PTEROYLGUTAMIC ACID ANTAGONIST ON THE RESPONSE OF THE AMPHIBIAN IMMATURE OVIDUCT TO ESTROGEN.* E. D. GOLDSMITH, SIDNEY S. SCHREIBER (by invitation), and ROSS F. NIGRELLI. (Department of Histology, New York University School of Dentistry and New York Aquarium, New York Zoological Society, New York, N.Y.)

Newly metamorphosed frogs (*Rana clamitans*) were treated parenterally with estradiol benzoate, pteroylglutamic acid (PGA), and 4-amino-pteroylglutamic acid (aminopterin). One group of animals received several dosage levels of aminopterin, or PGA, or both, and was simultaneously treated with the estrogen. A second group was pre-treated with aminopterin, PGA, or both, for 2 to 3 weeks, and then, in addition, was injected with estrogen for a period of 2 weeks. Grossly, the oviducts of the estradiol treated controls exhibited marked enlargement and coiling, whereas, the oviducts of the animals which received estradiol and aminopterin resulted in only slight enlargement and no coiling. Injections of estradiol for 2 weeks in animals pre-treated with PGA were followed by oviduct growth greater than that observed in estradiol controls. PGA in ratios of 100:1 of PGA to aminopterin showed but slight reversal of the antagonist effect. Histological observations confirmed the gross findings. Mitotic counts and their possible significance as to the site of action of the PGA antagonist in inhibiting growth in the presence of growth stimulating factor(s) will be discussed.

STUDIES ON THE MECHANISM OF ACTION OF AMINOPTERIN (4-AMINOPTERYLGLUTAMIC ACID) ON THE LYMPHATIC TISSUES

* Aided by a grant from the National Cancer Institute, National Institute of Health, U.S. Public Health Service.

* Work aided by a grant from National Cancer Institute, U.S. Public Health Service.

OF MICE. J. H. DOUGHERTY (by invitation), and T. F. DOUGHERTY. (Division of Oncology, Departments of Pathology and Anatomy, University of Utah College of Medicine, Salt Lake City, Utah)

Aminopterin (4-amino-pteroyl glutamic acid) is one of a group of substances which produces acute involution of lymphatic tissues. This compound, a folic acid antagonist, has received attention as a chemotherapeutic agent in acute leukemia. Since many agents produce lymphatic tissue involution through pituitary-adrenal cortical mediation, it was of interest to ascertain whether or not aminopterin acted through this mechanism.

Aminopterin in saline was administered intraperitoneally in dosages of 0.02 mg. daily to adrenalectomized and unoperated CBA mice. This amount is lethal for mice in approximately one week. After 5 days of treatment the animals were sacrificed and lymphatic tissues studied. A relative and absolute decrease in size of all lymphatic tissues was observed in the unoperated treated animals when these were compared with control mice of the same age. Involution of lymphatic tissues was not found in the adrenalectomized group although these animals lost as much weight as did the unoperated mice. A progressive absolute lymphopenia was observed in the unoperated animals after one day of treatment. A slight lymphopenia occurred in adrenalectomized animals after 5 days of treatment and there was an inhibition of the lymphocytosis characteristic of adrenalectomized mice. This amount of aminopterin failed to inhibit the growth of subcutaneous transplants of the Gardner lymphosarcoma (C3HED) although it produced acute involution of the lymphatic tissues of the tumor-bearing mice.

It is concluded that much of the effect of large doses of aminopterin on lymphatic tissues of mice is mediated through the adrenal cortex although there is evidence of some direct inhibitory action.

THE EFFECTS OF 4-AMINO-N¹⁰-METHYL-PTEROYL GLUTAMIC ACID AND 2,6-DIAMINOPURINE ON THE LEUKOCYTES OF THE NORMAL AND LEUKEMIC MOUSE.*
J. H. BURCHENAL, † J. L. BEIDLER, and J. NUTTING
(Introduced by C. P. Rhoads). (Section on Mouse Leukemia of the Division of Experimental Chemotherapy, of the Sloan-Kettering Institute for Cancer Research, New York, N.Y.)

In the screening of compounds for possible chemotherapeutic effects against transmitted mouse leukemia, 4-amino-N¹⁰-methyl-pteroyl glutamic acid and 2,6-diaminopurine have been found to be effective in prolonging the survival time of mice with the Ak 4 strain of leukemia. Since untreated mice injected with this strain

of leukemia show a marked increase in total leukocyte count and in the relative percentage of atypical prolymphocytes, it was felt worthwhile to investigate the effect of these compounds on the blood picture of normal and leukemic mice at therapeutic doses.

4-Amino-N¹⁰-methyl-pteroyl glutamic acid caused slight leukopenia in normal mice but there was no alteration of the differential count. 2,6-Diaminopurine, at these doses, had no significant effect on the total leukocyte count or differential of normal mice. The average leukocytic counts of leukemic mice receiving these drugs remained significantly lower than those of the leukemic controls after the ninth day of the experiment, and the differential counts done on the eleventh to the thirteenth day showed a suppression of the atypical prolymphocytes allowing a relative increase in the number of both mature lymphocytes and neutrophils. This suppression of the leukemic process with 4-amino-N¹⁰-methyl-pteroyl glutamic acid was also reflected in the microscopic appearance of section of liver, spleen, kidney, and bone marrow.

THE RESPONSE OF ACUTE LYMPHOID LEUKEMIAS IN MICE TO 4-AMINO-PTEROYL GLUTAMIC ACID (AMINOPTERIN). L. W. LAW and THELMA B. DUNN. (National Cancer Institute, Bethesda, Md.)

The effect of aminopterin has been studied on 4 transplantable lymphomas in the mouse. A detailed study of one of these, acute lymphoid leukemia, LI210, in the dba strain is presented. A statistically significant increase in survival time (approximately 40 per cent over controls), inhibition in the growth of localized leukemic mass, inhibition of infiltration into hemopoietic organs and maintenance of a normal blood picture, were found at a non-toxic optimal dosage level of 0.15 mg/kg. body weight.

The optimal dosage required for a similar response of the same leukemic cells grown in a different genetic background, DBF₁ mice was found to be 0.20 mg/kg. Aminopterin is effective in adrenalectomized mice at a somewhat reduced dosage. Synthetic folic acid, Diopterin and Teropterin did not effect the course of this leukemia in dba mice. Partial reversals of inhibition have been obtained with Teropterin at a ratio of 1:400.

Despite profound inhibition in growth and infiltration of leukemic cells, no histological differences in treated and normal leukemic cells have been noted and no toxic effects of the drug have been found in various tissues studied at the optimal dosage used. A reduction in reticulocytes to approximately 1/4 the normal mouse and to 1/6 the leukemic controls was noted.

TREATMENT OF ACUTE LEUKEMIA WITH A-METHOPTERIN (4-AMINO-N¹⁰-METHYL-PTEROYL GLUTAMIC ACID). LEO M. MEYER, (and by invitation) FRANKLIN R. MILLER, MANUEL J. ROWEN, GEORGE BOCK, and JULIUS RUTZKY. (Department of Therapeutics, New York University College of Medicine, New York, N.Y. and the Department of Medicine, Jefferson Medical College, Philadelphia, Pa.)

* This investigation was supported (in part) by a research grant from The National Cancer Institute of The National Institute of Health, U.S. Public Health Service, and (in part) by a research grant from The American Cancer Society.

† Fellow of The American Cancer Society, recommended by the Committee on Growth of The National Research Council.

Three children and 9 adults with acute leukemia were treated with a-methopterin (4-amino-N¹⁰-methylpteroyl glutamic acid). One child showed clinical improvement. Enlarged nodes and spleen were reduced in size. The leukocytes fell from 100,000 to 10,000/cu.mm. but blast cells persisted. A second child developed severe leukopenia with hypoplastic marrow. In a third child no change in the blood picture occurred and death followed massive hematuria, rectal bleeding, and oozing of blood from gums and lips. In the adult group one patient showed clinical improvement with reduction of total leukocytes from 100,000 to 19,000/cu.mm. but blast cells remained. In another adult there was clinical improvement on two occasions, with elimination of bone pain, subsidence of temperature, reduction of total leukocytes, and increase of neutrophiles. The bone marrow remained blastic. He developed exfoliative dermatitis of the hands and feet, bleeding from gums and lips, and ulceration of tongue and pharynx while under treatment. These cleared up after the drug was discontinued. Four patients showed no hematologic or clinical improvement but demonstrated toxic effects of the drug as manifested by dysphagia, nausea, vomiting, bowel hemorrhage and buccal mucosa ulcerations. Two cases developed severe leukopenia (200 W.B.C./cu.mm.) with severe hypoplastic marrow. Another patient developed a less severe leukopenia but the marrow remained hyperplastic and blastic. All 3 cases showed oral lesions and gastro-intestinal bleeding. In 2 of the 9 adults a deep blue hemorrhagic infiltration of the skin was observed. Anemia persisted in all of the patients observed.

THE INFLUENCE OF VARIOUS DIETARY FACTORS ON THE INDUCTION OF EPITHELIAL TUMORS IN MICE. H. P. RUSCH, R. K. BOUTWELL, and M. K. BRUSH (by invitation). (McArdle Memorial Laboratory, Medical School, University of Wisconsin, Madison 6, Wis.)

The influence of specific vitamins and of less clearly defined dietary factors on the formation of cancer has been the subject of a considerable number of reports. Many of these investigations were made before the influence of caloric restriction on tumor formation was known and before much information on the exact dietary requirements of the mouse was available. The present investigation is a report of the influence of various vitamins and other dietary factors on the induction by benzpyrene of epithelial tumors in mice. The caloric intake was carefully controlled.

The mice were divided into groups of 48 and the following diets were fed: (a) all vitamins high, (b) all vitamins low, (c) thiamine-riboflavin low, (d) pyridoxine low, (e) niacin-pantothenic acid low, (f) thiamine-riboflavin-pyridoxine-niacin-pantothenic acid low, (g) pteropterin substituted for folic acid, (h) highly purified diet, (i) rice diet, and (j) whole-wheat-milk diet. The vitamin level varied from minimal requirements to over one hundred times the required level in the high vitamin group. The final incidence of tumors was essentially the same in all groups except in group b, where the incidence was decreased by about 33 per cent. The condition of

the mice on all the groups remained good except for a few in group c which showed a thiamine deficiency and group b where signs of pyridoxine deficiency were noted in several mice. Such deficiencies were cured by the parenteral administration of the appropriate vitamin. The importance of controlling the caloric intake in such experiments is stressed.

ON THE STIMULATING EFFECT OF DIETARY FAT ON CARCINOGENESIS. R. K. BOUTWELL, H. P. RUSCH, and MIRIAM K. BRUSH (by invitation). (McArdle Memorial Laboratory, Medical School, University of Wisconsin, Madison 6, Wis.)

It is recognized that increasing the fat content of the diet tends to increase the rate of tumor formation. This phenomenon has not been adequately explained. At a fat level of 2 per cent, the incidence of epithelial tumors induced by benzpyrene in mice fed 6, 8, 10, and 12.1 calories per day was 19.5, 49, 70, and 82 per cent respectively. In the same experiment, mice fed a 28 per cent fat diet at the 6 and 10 calorie level had a tumor incidence of 30.5 and 76 per cent respectively. Forbes and coworkers have shown that there is a decreasing energy expense of utilization of the isocaloric intake of diets in the order of their increasing fat content. Using their data, it was calculated that the energy expense of utilization of the diet containing 2 per cent fat was about 3.1 calories, while the dynamic effect of 10 calories of the diet containing 28 per cent fat was about 1.6 calories. Thus the net energy value of the 28 per cent fat diet was greater by about 1.5 calories. By reference to a curve relating the tumor incidence to the caloric intake of a 2 per cent fat diet, it was seen that the degree of tumor stimulation due to the 28 per cent fat diet was of the same order of magnitude as that which resulted from an increase of 1.5 calories in the 2 per cent fat diet. It appears that the ability of fat to increase the net energy value of a diet is sufficient to explain the fat effect.

DEPENDENCE OF FORMATION OF SPONTANEOUS MAMMARY CARCINOMA IN MICE ON THE PROPORTION OF DIETARY FAT. ALBERT TANNENBAUM and HERBERT SILVERSTONE (by invitation). (Department of Cancer Research, Medical Research Institute, Michael Reese Hospital, Chicago 16, Ill.)

The formation of some types of tumors is accelerated by a "high" proportion of fat in the diet. It seemed worth while to examine the quantitative relationship between tumor formation and the degree of fat enrichment of the diet. For these studies the spontaneous mammary carcinoma was utilized. In one experiment employing 4 groups of 50 strain C3H mice, the proportions of dietary fat were 2, 6, 12, and 26 per cent of rations composed principally of Purina fox chow meal, skimmed milk powder, cornstarch, and Kremax (partially hydrogenated cottonseed-soybean oil). In another experiment employing 5 groups of 60 strain dba mice, the proportions of dietary fat were 2, 4, 8, 16, and 24 per cent of rations composed principally of casein, Kremax, cornstarch, salts, and B-vitamins. In both studies the pro-

portion of fat was increased by substituting Kremax for an equicaloric amount of cornstarch; protein, salts, and vitamins remained constant in amount. The diets were fed equicalorically at slightly below *ad libitum* levels. The experiments were continued until the mice were 2 years of age.

The results of the two investigations were in excellent agreement. It was found that the rate of tumor formation (as measured both by incidences and average times of appearance of the tumors) increased with increasing proportion of dietary fat. The effect was related specifically to the proportion of dietary fat and was not caused by different caloric intakes or different body weights of the mice.

THE EFFECT OF DIETARY FAT AND CARBOHYDRATE ON DIETHYLSТИLBESTROL INDUCED MAMMARY CANCER IN THE RAT.*

W. F. DUNNING, W. R. CURTIS, and M. E. MAUN
(by invitation). (Department of Pathology, Wayne University College of Medicine; Detroit Institute of Cancer Research; and St. Mary's Hospital, Detroit 1, Mich.)

The effects of dietary fat were assayed under conditions of controlled caloric intake by placing 84 AXC Line 9935 female rats with diethylstilbestrol pellets implanted in their scapular regions on isocaloric synthetic rations of varying fat and carbohydrate content. Diets adequate in protein, minerals and vitamins, varying in fat content from 6.5 to 46.0 per cent with sufficient dextrin to equalize the caloric content, were fed *ad libitum* and restricted to rats in individual cages. The caloric consumption varied from 40 calories daily for rats on the *ad libitum* high fat diet to 34 calories for those on the *ad libitum* low fat diet and their paired mates on the high fat diet, and was restricted to 25 calories in isocaloric portions of high fat, modified low fat, and low fat diets.

Of the 67 rats which survived for at least 180 days, 53 or 79 per cent developed 236 gross and 337 microscopic adenocarcinomas of the mammary gland. Restricting the caloric intake by 26 to 38 per cent of the *ad libitum* consumption did not decrease the percentage of rats which eventually developed mammary cancer, but increased the latent period from approximately 300 to 400 days.

More tumors were observed in a shorter average latent period in rats on a high fat diet than in their paired mates. Increased consumption of the high fat diet, however, lessened rather than enhanced these differences and the only consistent effect appeared to be an accelerated growth potential in the preformed cancer cells.

RELATION OF DIET TO THE DEVELOPMENT OF MAMMARY TUMORS INDUCED BY FEEDING 2-ACETYLAMINOFLUORENE.

R. W. ENGEL and D. H. COPELAND (by invitation). (Laboratory of Animal Nutrition, Alabama Polytechnic Institute, Auburn, Ala.)

Mammary tumors are consistently produced in female rats of the AES strain when subsisted on semi-synthetic diets containing 0.03 per cent 2-acetylaminofluorene (see Engel and Copeland, these meetings, 1948; and Science, 108:336-37, 1948).

Several diet modifications were employed to determine the relation of nutrition to the development of this type of tumor. Two basal diets were employed, differing in protein-content and protein-source. Basal diet C-1 contained approximately 11 per cent protein (9 per cent of casein and 20 per cent of degerminated corn grits) and basal diet C-20 contained approximately 20 per cent protein (6 per cent of casein and 30 per cent of alcohol-extracted peanut meal). Increasing the casein content of basal diet C-1 to 27 per cent resulted in more rapid body weight gains and an increase in consumption of the carcinogen. This, however, did not influence tumor incidence or time required for tumors to develop.

The addition of a detergent to basal diet C-20 at a level of 0.5 per cent appeared to enhance the action of this carcinogen; tumors appeared earlier, grew more rapidly and killed the host earlier when this diet modification was made. The addition of teropterin to basal diet C-1 (50 mg/kilo) likewise caused tumors to appear somewhat earlier. Varying the riboflavin content of basal diet C-1 (from 1 to 100 mg/kilo) failed to influence tumor incidence or induction time.

THE OCCURRENCE OF NEOPLASMS IN CHICKENS AS A RESULT OF PROLONGED CHOLINE DEFICIENCY. A. E. SCHAEFER (by invitation), D. H. COPELAND (by invitation), and W. D. SALMON (by invitation) (Introduced by R. W. ENGEL). (Laboratory of Animal Nutrition, Alabama Polytechnic Institute, Auburn, Ala.)

Cancer was observed in 12 of 23 chickens that died while receiving diets low in choline. No tumors were found in seven control chickens receiving the same diets supplemented with 0.3 per cent choline chloride for comparable periods of time. This extends to another species, the previous results on the production of neoplasms in rats. (Am. J. Path., 22:1059, 1946, Annal New York Acad. Sci., 49:49, 1947.) Two diets, similar to those in the rat studies except for the vitamin and mineral modifications necessary for chickens, were used. At the beginning of the experiment the diets were supplemented with 0.1 to 0.3 per cent choline chloride which was reduced to 0.05 per cent or zero after 13 to 23 weeks. Neoplasms were observed after the birds received the low choline diets for 14 to 44 weeks or a total of 33 to 60 weeks on experiment. A total of 16 tumors were identified; three of these were classified as metastases. Adenocarcinoma of the liver was observed in 3 chickens and in two of these there were metastases to the intestine. One chicken had a hemangio-endothelioma of the liver with a metastasis to the ovary. Cholangioma of the liver was found in one bird. One fibromyxoma and two embryonal nephromas were observed in the kidneys of 3 chickens. Five subcutaneous tumors were found. Four of these were fibrosarcomas and one was a benign fibroma.

* Supported in part by a grant-in-aid from the U.S. Public Health Service.

PIOSPHAMIDASE IN NEOPLASMS. GEORGE GOMONI. (Dept. of Medicine, University of Chicago, Chicago 37, Ill.)

Results obtained with the new histochemical reaction for phosphamidase (Proc. Soc. Exper. Biol. and Med., In press) are presented. This enzyme occurs in small to moderate amounts in all tissues but in large amounts only in the gray matter of the central nervous system and in malignant epithelial tumors. In over 60 carcinomas of all kinds intense differential staining of the malignant change, setting it off against its environment, was obtained. The intensity of the reaction generally parallels morphologic criteria of malignancy, although sometimes fairly intense reactions are obtained in rectal polyps, not malignant morphologically. In sarcomas the results are variable; some of them stain just like carcinomas, while others show a patchy reaction or none at all.

**THE EFFECT OF CERTAIN STEROIDS ON SUC-
CINOXIDASE, AND GLYCOLYSIS OF TU-
MOR SUBCELLULAR (PARTICULATE)
FRACTIONS.** KENT WIGG (by invitation), and DEAN BURK. (National Cancer Institute, Bethesda, Md.)

It has been found that certain hormones act competitively on the succinoxidase system of the mitochondrial elements of the S91 and Harding-Passey melanomas grown in dba and C mice respectively. The mitochondrial elements (microscopically visible cytoplasmic particulates, mostly melanized; see Woods, Du Buy, Burk, Hesselbach, and Lackey, in J. Nat. Cancer Inst., Feb., 1949) were obtained by centrifugation at 10° C. of saline, glass wool-filtered tumor homogenates at 10,000g for 30 minutes after preliminary centrifugation at 25° for 10 minutes to eliminate nuclei and heavy phagocytized cell material. The tumors used for studies of glycolysis were homogenized in isotonic sucrose. Oxygen consumption was measured manometrically after addition of 0.05M phosphate (pH 7), 0.00001M cytochrome c, and 0.02M succinate. Glycolysis was measured by CO₂ liberation from a system consisting of a nutrient solution (made according to Le Page, J. Biol. Chem., 176:1021-27, 1948) and the sterol.

Diethylstilbestrol, at a concentration of ~ 135/ml. ($\approx 0.0005M$), inhibited particulate oxygen consumption 60 to 75 per cent. This inhibition was markedly reduced or entirely eliminated by the simultaneous addition of ~ 125/ml. ($\approx 0.0002M$) progesterone or testosterone. Progesterone alone had no effect on oxygen consumption. S91 and Harding-Passey tumor particulates behaved similarly except for differences in the oxygen uptake. Diethylstilbestrol also markedly inhibited or eliminated oxygen consumption by mitochondrial elements from tumors such as the C3HBA breast carcinoma and sarcoma 37 grown in C3H and C mice respectively.

Anaerobic glycolysis was stimulated 50 per cent in the case of the C3HBA breast carcinoma by diethylstilbestrol.

β -GLUCURONIDASE ACTIVITY AND LACTIC ACID CONTENT OF BODY FLUIDS OF PATIENTS WITH AND WITHOUT CANCER.* WILLIAM H. FISHMAN, RICHARD L. MARKUS, and PAUL H. PFEIFFER (by invitation), and F. HOMBURGER. (Laboratories of the Cancer Research and Cancer Control Unit of the Department of Surgery and the Departments of Biochemistry and Medicine, Tufts College Medical School, Boston, and the Department of Clinical Research, Jewish Memorial Hospital, Roxbury, Mass.)

Ordinarily those constituents of neoplastic exudates which derive from the metabolism of tumors are not in equilibrium with the same substances in the blood, because of their more rapid genesis in the tumor and entry into the exudate, and because of the slow rate of return of such components into the circulation. Therefore, it is reasonable to expect that the composition of pleural and ascitic fluids occurring in the presence of malignancies would reflect the biochemical changes peculiar to the tumors. Substances in such fluids which occur in higher or lower concentration than in the blood thus are of metabolic interest in the study of malignancy.

As a part of a systematic investigation on the significance of the chemical composition of fluids and secretions in human cancer, the β -glucuronidase activity and the lactic acid content of a number of body fluids have been studied. Measurements of β -glucuronidase activity were chosen because of the previous observations of one of us (W. H. F.), which indicated the presence in the majority of cases studied of higher levels of β -glucuronidase in cancer tissue than in the tissue of origin. Lactic acid was determined because of the well-established ability of tumor tissue to produce lactic acid from glucose by anaerobic glycolysis. Wherever possible the sediment of the fluid prepared by centrifugation was stained according to the Papanicolaou technique and studied microscopically. Pathological confirmation was obtained, in addition, by autopsy in a high percentage of cases.

In a significant number of fluids from patients with carcinomatosis of the chest or abdominal cavities, both the glucuronidase and the lactic acid values were considerably elevated. In some, either one or the other showed abnormal values, and in others neither was abnormal. Determination of lactic acid by quantitative chemical methods was found essential, the usual clinical qualitative methods being entirely unreliable. Chemical and enzymological observations have been correlated with the clinical status of the patient, especially with regard to previous therapy. The considerations which led to this work on fluids contained in closed body cavities apply in part also to body secretions and the same studies are being applied at present in this laboratory to gastric juice and vaginal secretions. The possible diagnostic value of such determinations cannot of course be ascertained until sufficient comparative studies have been made on secretions, transudates and exudates in patients without cancer.

* This work was aided by an institutional grant of the American Cancer Society and by a research grant from the National Cancer Institute of the National Institute of Health, U.S. Public Health Service.

AN ANALYSIS OF THE β -GLUCURONIDASE CONTENT OF CIRCULATING WHITE BLOOD CELLS OBTAINED FROM HUMAN LYMPHOBLASTOMATOUS AND CONTROL WHOLE BLOOD SAMPLES. A. JOHN ANLYAN and JESS F. GAMBLE (by invitation), and HERMAN A. HOSTER. (Ohio State University College of Medicine, Columbus, Ohio)

The work of Fishman and Anlyan has demonstrated a greater than normal content of the enzyme β -glucuronidase in human neoplastic tissues. It has further been shown that the enzyme β -glucuronidase is present for the most part in the buffy coat of human blood. The purpose of the present study is a quantitative estimation of the β -glucuronidase content of the buffy coat of human Hodgkin's disease and leukemic bloods. As a control measure, a number of determinations using the blood of normal human subjects and patients with various malignant conditions other than lymphomata were made.

A new method for separation of the buffy coat from the other elements of human whole blood has been devised by the authors. The β -glucuronidase activity per gram of buffy coat in patients with Hodgkin's disease, lymphatic leukemia, monocytic leukemia, and myelogenous leukemia is determined with repeat determinations during and after therapy. The results at the present time indicate that the buffy coat β -glucuronidase activity of patients with the leukemias and Hodgkin's disease varies significantly from the activity observed in the normal control group.

ACID PHOSPHATASE ACTIVITY OF THE GASTRIC CONTENTS OF PATIENTS WITH CARCINOMA OF THE STOMACH. CHARLES E. DUNLAP and GEORGE W. CHANGUS (by invitation). (Department of Pathology, School of Medicine, Tulane University and Charity Hospital, New Orleans, La.)

In patients with carcinoma of the stomach histochemical studies by Gomori have shown high acid phosphatase activity in the neoplasm and also in the surrounding gastric mucosa. Chemical determinations for acid phosphatase were done by the method of Gutman and Gutman on gastric contents, aspirated from a series of fasting patients with and without carcinoma of the stomach. It was found that aspirates having an initial pH of less than 3.5 seldom contained significant amounts of acid phosphatase and that the enzyme, when present in other samples, could be irreversibly inactivated, *in vitro*, by acidification to pH 3.5 or less. At a pH greater than 3.5 the enzyme was fairly well preserved for 48 hours at 4° C. but rapid deterioration occurred at room temperature. Thus gastric aspirates containing "free acid" (pH less than 3.5) as well as those that had stood for more than 2 hours without refrigeration were considered unsuitable for acid phosphatase determinations. In a great majority of the patients with carcinoma of the stomach no "free acid" was present and the aspirates were found to contain more than 10 units of acidphosphatase per hundred cc. Most samples from patients

without gastric carcinoma contained less than 10 units. The study, to date, has covered only a limited number and variety of gastric lesions and includes no cases of early carcinoma.

PURINE METABOLISM IN TETRAHYMENA AND ITS RELATION TO NEOPLASTIC TISSUE. G. W. KIDDER, VIRGINIA C. DEWEY, and R. E. PARKS, JR., GILBERT L. WOODSIDE (Introduced by J. C. Aub). (Biological Laboratory, Amherst College, and Zoological Laboratory, University of Massachusetts, Amherst, Mass.)

The ciliated protozoan, *Tetrahymena geleii*, has been shown to have a requirement for the purine, guanine, or its riboside. A series of purine analogues has been tested as inhibitors of purine metabolism. Of 16 analogues tested, 6 proved to have an inhibitory effect. Of these six, 4 were xanthine analogues. The inhibition indices were: caffeine, 100; theobromine, 150; theophylline, 225; and paraxanthine, 300. None of these inhibitions was completely reversed by guanine. The remaining compounds, 5-amino-7-hydroxy-1-v-triazolo-(d)-pyrimidine and 5,7-diamino-1-v-triazolo-(d)-pyrimidine had indices of 0.075 and 85 respectively.

Recent tests on another series of twelve analogues showed that 2,6,8 triamino purine had an inhibition index of about 15. On the other hand, 2,4 diamino-5-formyl-amino-6 hydroxypyrimidine spared guanylic acid. This demonstrates the ability of the organism to synthesize guanine and/or adenine by completing the imidazole ring. It appears that water is split out from the adjacent aldehyde and amino groups in the 5 and 4 positions respectively. Acting on the assumption that neoplastic tissue differs from normal animal cells in its guanine requirement, the most effective compounds of this series were tested. Positive results of these tests which have been obtained will be reported.

THE APPLICATION OF DIFFERENTIAL CENTRIFUGATION AND ELECTRON MICROSCOPY TO THE SEGREGATION AND VISUAL STUDY OF HUMAN LYMPH NODE CELL MACROMOLECULAR PARTICLES IN THE SIZE RANGE OF 20 TO 300 m μ . MIRIAM S. HOSTER, BETTE J. MCBEE, and HARRY A. ROLNICK (by invitation), and HERMAN A. HOSTER. (The Ohio State University College of Medicine, Columbus, Ohio)

Since no information concerning the segregation and visual study of human lymph node cell macromolecular components in the size range of 20 to 300 m μ was available in the literature, the present study was undertaken to develop a satisfactory technique for this purpose. Differential centrifugation was used to separate these lymph node cell components obtained from patients with Hodgkin's disease and control diseases of diverse etiology. After each step in the separation procedure, a study of these bodies was made in the electron microscope.

The research tools utilized in this study were the ultra-centrifuge, the dark field microscope and the elec-

tron microscope. Chemical and spectrophotometric studies were included as a supplementary guide in component separation. A number of separation techniques will be presented with the significant factors involved in each. These considerations include the type of diluent used, the hydrogen ion concentration, the duration of the extraction period at 4° C. and the methods of differential centrifugation. Calcium chloride and osmium vapor staining of formvar coated electron microscope screens whose surfaces are covered with a fine film of the sample to be studied will be discussed. A description of the preliminary observations recorded to date is included.

EFFECT OF INOCULATION OF THE VIRUSES OF INFLUENZA, HERPES, AND RUSSIAN FAR EAST ENCEPHALITIS ON THE GROWTH OF TRANSPLANTABLE TUMORS IN MICE. ALICE E. MOORE (Introduced by C. P. Rhoads). (Division of Experimental Chemotherapy, Sloan-Kettering Institute, New York, N.Y.)

Three viruses of widely different characteristics were studied for their ability to grow in transplantable mouse tumors and to determine the effect of such a parasitization in the viability of the tumor.

It was found that both the viruses of influenza and herpes could grow for a limited period when inoculated directly into sarcoma 180. Their presence had no effect on the growth or transplantability of the tumor. There was no evidence that these viruses had any special affinity for either sarcoma 180 or the mouse adenocarcinoma EO 771.

In contrast, the virus of Russian Far East Encephalitis not only rapidly parasitized sarcoma 180 but showed a definite preference for the neoplasm. In the process of viral infection tumor growth was definitely inhibited. Complete destruction of the tumor, which could be demonstrated by bioassay into virus immune mice and by cytological study was always associated with systemic infection and death of the animal.

ISOLATION OF THE MOUSE MAMMARY CARCINOMA VIRUS. SAMUEL GRAFF, WENDELL M. STANLEY, DAN H. MOORE, and HENRY T. RANDALL (by invitation), and CUSHMAN D. HAAGENSEN. (College of Physicians and Surgeons, Columbia University, New York, and Rockefeller Institute For Medical Research, Princeton, N.J.)

Characteristic sub-microscopic particles have been isolated from milk of high cancer strain mice. This material could not be detected in the milk of a cancer free strain. Minute amounts of this material produce carcinoma in otherwise cancer-free mice. Similar particles were isolated from the milk of low cancer strain mice after they were foster-nursed on high cancer strain mothers.

AN IMPROVED METHOD FOR THE STUDY OF CERTAIN METABOLITES OF THE CARCINOGENIC AZO DYES. NELSON F. YOUNG. (Samis Grotto Cancer Research Laboratory, Medical College of Virginia, Richmond, Va.)

One phase of investigation of the problem of butter-yellow carcinogenesis in rats has been the demonstration of certain metabolites of the dye *in vivo* and *in vitro*. The methods by which such metabolites have been isolated and quantitated are somewhat tedious and require the use of special reagents and equipment. By an adaptation of the technique of paper chromatography, such separations may be made conveniently and quickly. The method requires no special equipment or reagents and the sensitivity compares favorably with existing methods. Since the separations are followed visually, the modifications necessary for the isolation of new compounds or work on dyes other than butter-yellow are rapidly and readily made. A comparison of the *in vitro* metabolites of several carcinogenic and closely related non-carcinogenic dyes will serve to demonstrate the usefulness and limitations of the method.

DISTRIBUTION OF PHOSPHORUS-CONTAINING COMPOUNDS IN MAMMARY GLANDS AND MAMMARY TUMORS OF MICE BY RADIOPHYSICAL METHODS. S. ALBERT, and RALPH M. JOHNSON, and PATRICIA J. KELLER (by invitation). (Richard Cohn Radiobiology Laboratory of the Detroit Institute of Cancer Research, Detroit 1, Mich.)

An attempt has been made to determine some of the changes in the metabolism of phosphorus-containing compounds accompanying mammary cancer development. Radioactive phosphorus was injected into tumor-bearing high cancer strain and into non-tumor bearing low cancer strain female mice; 17 hours later they were sacrificed. Phosphorus-containing compounds of mammary glands and tumors were separated by a method modified from those of Schneider and Schmidt, and Thannhauser.

The phosphorus in tumors was evenly distributed between the acid-soluble, desoxyribonucleic and ribonucleic acid fractions, and was lowest in the phospholipid. In mammary glands the phosphorus was highest in the acid-soluble fraction, lower in the phospholipid, and lowest in the ribonucleic and desoxyribonucleic acids.

The radioactivity was found to have the same general distribution in mammary glands as in tumors. There was a definite shift, however, of radioactivity from the acid-soluble to the acid-insoluble fraction in tumors. This was evident in the desoxyribonucleic and ribonucleic acids but not in the phospholipid. At this interval after injection, the radio phosphorus per microgram of phosphorus was about twice as high in the tumor desoxyribonucleic acid, ribonucleic acid, and phospholipid fractions as in the corresponding fractions of non-cancerous mammary glands. The uninvolved mammary glands of tumor-bearing cancer susceptible females contained more phosphorus on a wet weight basis than did those of cancer resistant animals. This difference disappeared, however, when expressed on the basis of total nitrogen, probably indicating more glandular tissue per unit weight in the mammary glands of cancer susceptible animals.

AGE AND STRAIN DIFFERENCES IN PHOSPHORUS METABOLISM IN VARIOUS ENDOCRINE ORGANS OF MICE. RALPH M. JOHNSON (by invitation), and S. ALBERT. (Richard Cohn Radiobiology Laboratory of the Detroit Institute of Cancer Research, Detroit 1, Mich.)

The metabolic activity of endocrine glands in high and low cancer strains of mice may shed light on the relationship of endocrine activity to the process of carcinogenesis. The important role of phosphorus in cellular metabolism suggests that the uptake of this element (as radioactive phosphorus) can be used as a measure of the functional activity of such endocrine glands.

Mice of the C57 and dba strains were injected with radioactive phosphorus (P^{32}) as inorganic phosphate, and killed at intervals up to 48 hours following the injections. Pituitaries, thyroids, adrenals, testes, and blood were removed, digested, and aliquots taken for both radioactivity assay and colorimetric determination of P^{31} .

Based upon accumulation of radioactive phosphorus, the testes of both strains were the least active followed by the pituitaries, thyroids, and adrenals, increasing in that order. Age variations did not alter this relationship. The adrenals of the immature dba mice were more active than those of C57 mice of a comparable age. The reverse was true, however, in the mature animals. The testes of the immature animals of both strains accumulated phosphorus to the same extent. In the older animals, the phosphorus uptake by testes in C57 mice exceeded that found in the mice of the dba strain. Within either strain, the adrenals and testes of the immature animals accumulated more phosphorus than the same organs of the mature animals. The uptake of radiophosphorus by pituitaries and thyroids appeared to be independent of both the age and strain of the animals.

LOCALIZATION OF RADIOACTIVE COMPOUNDS IN TUMORS. WM. G. MYERS. (College of Medicine, The Ohio State University, Columbus, Ohio)

Several hundred intermediate compounds are being collected and purified, or synthesized, to which are coupled radioactive sulfanilic acid labelled with S^{35} and iodinated derivatives and isomers of it labelled with I^{131} , by azo linkages. Most of the products are radioactive acidie or basic dyes of widely varied structures which contain several functional groups. Purification of these is readily carried out by chromatographic adsorption methods.

A rapid scanning procedure has been evolved for the comparison of the relative concentrations of radioactivity in the liver, spleen, kidney, skeletal muscle, blood, and the transplanted tumors, C3HBA adenocarcinoma, 15091a spindle cell mammary carcinoma, and sarcoma 37 in C3H, ABC, and CFW mice respectively, at 15 minutes, 1 hour, 4 hours, and 25 hours after the intravenous injection of approximately 1 mg. of each of the radioactive compounds.

Two of the forty S^{35} labelled compounds tested to date have shown concentrations of radioactivity in the

carcinomas up to double those in the blood and five times the concentrations in the muscles at one or more of the intervals after injection. More extensive tests on these compounds and derivatives and isomers of them are in progress.

THE DISTRIBUTION OF RADIOACTIVITY AND THE METABOLIC DEGRADATION IN THE MOUSE OF 20-METHYLCHOLANTHRENE-11-C¹⁴. WILLIAM G. DAUBEN and DOROTHEA MABEE (Introduced by HARDIN B. JONES). (Department of Chemistry and Radiation Laboratory, University of California, Berkeley, Cal.)

These experiments were undertaken to study the distribution and metabolism of methylcholanthrene and to compare these findings with the dibenzanthracene results of Heidelberger and Jones.

Strain A male mice were injected with methylcholanthrene-C¹⁴. Radioactivity was found in all tumors and in 24 per cent of the tumors more than 5 per cent of the injected dosage was present. The amount of methylcholanthrene injected had no effect upon the percent of activity found in the tumor. The tumors were then subjected to chemical analysis. The amount of methylcholanthrene which was found in the tumor was of the same order of magnitude as that reported in the dibenzanthracene study. It was also found that the distribution of the degradation product of methylcholanthrene followed the same pattern as dibenzanthracene. Since the metabolism of methylcholanthrene and dibenzanthracene is similar and whereas the carcinogenic index of methylcholanthrene is three times that of dibenzanthracene, it can be concluded that methylcholanthrene is three times more active and metabolizes three times more rapidly.

Site of injection, liver, fat, and muscle were removed at intervals for rate studies. At the end of 7 weeks, 50 per cent of the injected dose had disappeared from the injection site. Activity in liver, fat, and muscle was absent or negligible. The rate of elimination in urine and feces was determined for the first week following injection, and totally 1 to 3 per cent of the injected dose was eliminated in the urine and approximately 10 per cent in the feces.

USE OF STARCH COLUMN CHROMATOGRAPHY IN STUDY OF AMINO ACID COMPOSITION AND DISTRIBUTION OF RADIOACTIVITY IN PROTEINS OF NORMAL RAT LIVER AND HEPATOMA. PAUL C. ZAMECNIK, and IVAN D. FRANTZ, JR., and MARY L. STEPHENSON (by invitation). (From the Medical Laboratories of the Collis P. Huntington Memorial Hospital of Harvard University at the Massachusetts General Hospital, Boston, Mass.)

Animal tissue is composed of many proteins of varying amino acid composition. In the synthesis of these proteins and the direction of the sequential arrangement of the peptide bound amino acids it is likely that a number of highly specific enzymes participate. The possibility thus suggests itself that a change might occur in the proteosynthetic enzymes of malignant tissue which

would be reflected in an altered amino acid composition of the proteins synthesized. By means of starch column chromatography, and with the help of Drs. Stanford Moore and William Stein, comparisons have been made of the amino acid composition of trichloroacetic acid precipitated proteins obtained from primary rat hepatomas and from normal rat livers. Chromatograms from the normal and malignant tissues have been found to be in most respects superimposable. A small but significant increase in the leucine-isoleucine peak has, however, been found in the hepatoma.

In a series of *in vitro* experiments, C^{14} -carboxyl-labeled alanine, C^{14} -carboxyl-labeled glycine, and $\text{NaHC}^{14}\text{O}_3$ have been added to tissue slices of normal liver and of hepatoma. Starch column chromatograms have been made of hydrolysates of the slice proteins at the end of the experiments. In the $\text{NaHC}^{14}\text{O}_3$ experiments, radioactivity has been found predominantly in aspartic and glutamic acids, and in arginine. Smaller amounts of radioactivity have also been found in glycine and serine, indicating carbon dioxide fixation in these amino acids in a manner as yet unexplained and heretofore unknown. Preliminary experiments suggest greater fixation of carbon dioxide in glycine in the hepatoma than in the normal liver slice. The fixation of carbon dioxide in the guanidine group of arginine is decreased in the hepatoma.

AMINO ACIDS IN HYDROLYSATES OF THE MITOCHONDRIAL FRACTION IN NORMAL AND NEOPLASTIC TISSUES AS STUDIED BY PAPER CHROMATOGRAPHY. EUGENE ROBERTS and CHAO-T'E LI (by invitation). (Department of Anatomy, Division of Cancer Research, Washington University Medical School, St. Louis, Mo.)

Mitochondria were isolated from normal mouse liver, pancreas, and kidney and from mouse mammary and squamous cell carcinomata and hepatoma by differential centrifugation according to the method of Hogeboom *et al.* The isolated particles were the same size and shape as the structures identified as mitochondria in smears made from homogenates and in free cells found in the sediment from the first low-speed centrifugation, and they possessed the same staining characteristics with Janus green B before fixation and with aniline-acid fuchsin after fixation with osmic acid.

Aspartic acid, glutamic acid, glycine, alanine, valine, the leucines, serine, and proline were the amino acids present in greatest quantities after hydrolysis of the mitochondria for 24 hours in 6N HCl in a sealed tube at 109° C. Threonine, tyrosine, phenylalanine, cystine, histidine, and arginine were present in considerably smaller quantities. The results indicate a large excess of the dicarboxylic amino acids over the basic amino acids. The patterns found for the different tissues will be shown and the similarities and differences in the normal and malignant tissues will be discussed.

AMINO ACIDS IN CERTAIN NORMAL AND NEOPLASTIC TISSUES. H. E. SAUBERLICH and C. E. BLADES (by invitation), and C. A. BAUMANN.

(Department of Biochemistry, University of Wisconsin, Madison 6, Wis.)

Representative normal and tumor tissues were hydrolyzed with acid or alkali for various periods of time and the hydrolysates analyzed microbiologically for 18 amino acids. The tumors included the Flexner-Jobling carcinoma, spontaneous mammary adenocarcinoma of the rat, sarcomas of the rat induced by methylcholanthrene, and hepatomas induced by azo dyes. The normal samples included muscle and liver from normal rats, and liver from rats fed restricted amounts of food or fed a non-carcinogenic azo dye.

Each of the 18 amino acids was found in every sample analyzed. The amounts ranged from 12 per cent for glutamic acid (calculated to 16 per cent N in the moisture-free, fat-free, residue) to approximately 1 per cent for tryptophan and cystine. All other amino acids were present in intermediate amounts. For most amino acids optimal amounts were found after 3 to 8 hours of hydrolysis. In general the percentages of the individual amino acids found in the tumor samples did not differ greatly from those found in normal rat liver or rat muscle, or from the values reported by others for representative cuts of beef or pork.

However, the spontaneous mammary tumors of the rat proved to be exceptional in that they contained abnormally high amounts of glycine (17.5 per cent) and proline (10 per cent) and relatively low amounts of histidine and methionine. The discrepancy is attributed to the connective tissue present in the latter tumors.

THE EFFECT OF COBALT ON THE CORRELATION OF NUCLEIC ACID CONCENTRATION WITH RATE OF GROWTH. HILTON LEVY, ELIZABETH SKUTCH, and ARTHUR L. SCHADE (introduced by DEAN BURK). (Overly Biochemical Research Foundation, Inc., New York, N.Y.)

The concentration of pentose nucleic acid (PNA) and of desoxypentose nucleic acid (DNA) has been studied as a function of the rate of growth of *Proteus vulgaris*. During early logarithmic growth of the organism in meat extract broth, the concentration of PNA whose initial value ranged from 6 to 12 per cent of the dry weight, increased two to three fold. The concentration of PNA followed closely the synthetic activity of the culture, expressed as per cent increase in dry weight per hour. The DNA concentration varied (3 to 6 per cent on dry weight basis) over the period of growth studied and showed no correlation with the growth rate.

Cells of *Proteus vulgaris*, inoculated into meat extract broth containing growth-inhibitory concentrations of cobalt, behaved like resting cells in that they did not increase in size, nor divide, nor show the increased Q_{O_2} characteristic of normal growing cells. The changes in PNA concentration, on the other hand, followed a course that was essentially identical to that of growing bacteria, i.e. a 2 to 3 fold increase in PNA concentration occurred at the same rate as in the control cultures. Such increase in dry weight of the culture as was found over the period of observation could be accounted for largely by the increase in the amount of PNA. The DNA

concentration of the cobalt inhibited cells varied in a manner similar to that of the control culture.

APPLICATION OF CHROMATOGRAPHY TO THE SEGREGATION OF SUBCELLULAR PARTICULATES. VERNON T. RILEY (by invitation), MARIE L. HESSELBACH, M. W. WOODS, and DEAN BURK. (National Cancer Institute, Bethesda, Md.)

Melanized granules of the Cloudman S91 and Harding-Passey mouse melanomas can be reversibly adsorbed on cellulose columns and are thus subject to chromatographic manipulation. As a consequence, certain other constituents of the tumor homogenates can be readily separated from the granules, thereby providing a basis for non-centrifugal segregation of a substantial portion of the other tissue components. The granules so separated appear essentially as a homogeneous population in the phase contrast microscope. Granules of the Harding-Passey tumor examined in the electron microscope were also essentially free of contaminating microsomal elements and particulate debris. The mean diameter of these chromatographed granule preparations was 0.3 to 0.4 microns, with a size range of approximately 0.2 to 0.6 microns.

The particulates separated from the S91 melanoma by chromatography were studied enzymatically with respect to succinoxidase and dopa oxidase with a $QO_2(N)$ increase of approximately 6 fold and 10 fold respectively when compared to the starting extract partially purified by centrifugal clearance.

The adaptation of chromatography to subcellular particulates ranging from the chicken tumor agent in the virus range to melanized granules in the mitochondrial and bacterial range provides another method of separating and characterizing intracellular particulates.

PHYSICAL CHEMICAL STUDIES ON SERA OF MYELOMA PATIENTS.* KURT G. STERN (by invitation), DANIEL LASZLO, and JOSEPH S. KRAKAUER (by invitation). (Dept. of Chemistry, Polytechnic Institute of Brooklyn and Division of Neoplastic Diseases, Montefiore Hospital, New York, N.Y.)

The examination of the sera from 7 cases of multiple myeloma in the electrophoresis apparatus, using the Svensson optical system, revealed in 6 cases the presence of a large amount of protein component migrating with the mobility of gamma globulin. Five of the patients exhibited a hyperproteinemia, with a total protein concentration ranging from 8.5 to 14 per cent, while 2 sera showed a normal total protein content. One of the latter sera yielded a nearly normal electrophoresis diagram while the other was found to have a low albumin and a high gamma globulin concentration.

The protein fraction of lowest mobility was isolated by preparative electrophoresis from the serum of a patient in which it amounted to 70 per cent of the total

protein present. The purified protein fraction was further characterized by measuring the sedimentation rate in the analytical ultracentrifuge, employing the Philpot optical system ($s_{20} = 6 \times 10^{-13}$); the rate of free diffusion in a Claesson cell, using the schlieren-scanning method of Longsworth, the relative viscosity in a capillary viscosimeter by a photographic recording method ($\eta_{sp}/c = 0.1$), as well as the ultraviolet absorption spectrum in a Beckman quartz spectrophotometer (ϵ_{max} at 278-282 μm), were also determined.

The outstanding property of the protein studied by us appears to be its remarkable ultracentrifugal paucity or monodispersity and its electrophoretic homogeneity which serve to distinguish the material from normal human gamma globulin.

CALCIUM AND POTASSIUM CONTENT OF REGENERATING TISSUES. DALE REX COMAN, and (by invitation) ROBERT P. DELONG, and I. ZEIDMAN. (Department of Pathology, University of Pennsylvania, School of Medicine, Philadelphia 4, Pa.)

Observations by others have revealed a local deficiency of calcium in cancerous tissue. In our laboratory the calcium deficiency of cancer cells has been related to a decreased adhesiveness of these cells, which in turn we have related to the invasive propensities of malignant tumors. The present preliminary study was an attempt to determine whether calcium deficiency is always associated with a state of active cell multiplication, or whether its existence in cancerous tissue is dependent upon some other features of the neoplastic state. The material used consisted of normal and regenerating rat livers. Flame photometric determinations of the calcium, sodium, and potassium content of these tissues were made. No significant differences were found in the calcium content, although regenerating tissue had a higher potassium content than the normal. This result is consistent with the hypothesis that the low calcium content of cancer tissue depends upon some other property than the rate of cell multiplication.

MINERAL METABOLISM IN METASTATIC BONE CANCER.* DANIEL LASZLO. (Division of Neoplastic Diseases, Montefiore Hospital, New York, N.Y.)

Patients suffering from cancer metastases to the bones were studied under controlled metabolic conditions. Calcium, phosphorus, and nitrogen balances were determined in patients with predominantly osteolytic and osteoblastic metastases respectively. The chemical data were correlated with the clinical course and with the therapeutic measures.

Hypercalcemia is frequently associated with metastatic malignancy. Among 70 breast cancers with bone metastases, studied within one year, 10 were found to have hypercalcemia. The importance of early recognition and early treatment of this complication is illustrated and the metabolic data are presented. Osteolytic

* (This investigation was supported (in part) by a research grant from the American Cancer Society and the National Cancer Institute of the National Institute of Health, U.S. Public Health Service.)

* This investigation was supported by a research grant from the American Cancer Society.

metastases appear to be characterized by high urinary calcium concentration, high urinary calcium output and a negative calcium balance. In osteoblastic metastases secondary to prostatic malignancy a low calcium concentration in the urine, a low urinary calcium output and calcium retention were observed. The effect of androgens and estrogens on the mineral metabolism of such patients is illustrated.

THE SOURCE OF TUMOR NITROGEN IN RATS BEARING WALKER CARCINOMA 256. CHARLES D. SHERMAN, JR. (by invitation), and G. BURROUGHS MIDER. (Department of Surgery, The University of Rochester School of Medicine and Dentistry, Rochester 7, N.Y.)

Earlier experiments in our laboratory indicated that the nitrogen content of Walker carcinoma 256 exceeded the amount of dietary nitrogen stored by the host during the period of tumor growth. Therefore, most of the nitrogen in the tumor must have been obtained from the rat's tissues. The experiment to be reported was designed to show which tissues contributed nitrogen to the neoplasm.

The wet and dry weights and nitrogen content of the livers, spleens, lymph nodes, kidneys, and hearts were determined in 20 rats bearing the Walker tumor and in pair fed normal controls of the same age, weight, and sex. The remainder of each carcass ("residue") was analyzed for nitrogen content. The results indicate that most of the tumor nitrogen was contributed by the carcass "residue." The lymph nodes of the tumor-bearing rats lost 50 per cent more nitrogen than did the pair fed controls while no significant differences were found in the weights or nitrogen content of the spleen and kidneys in the two groups. The heart gave up nitrogen only in the rats with the largest tumors. The livers of the tumor-bearing rats, however, contained significantly more nitrogen than did the livers of the pair fed controls.

MELANOMA IN DOGS. R. M. MULLIGAN (Introduced by H. L. STEWART). (University of Colorado Medical Center, School of Medicine, Denver, Colo.)

Of 31 dogs with 36 melanomas (17 non-cancerous and 19 cancerous), the sex (19 male and 11 female) and age (6 to 14 years in 27) were known in 30. The breeds affected usually had deeply or moderately pigmented skin. The primary neoplasm was found on the head (8 on the eyelids and 4 in the cheeks), the thorax, and the extremities in 29 cases. The smaller tumors (less than 6 cc. in volume) were often non-cancerous and the larger (greater than 8 cc. in volume) were often cancerous. The characteristics of the neoplastic cells and of their growth pattern, the tendency to invade, and the metastasis were helpful in differentiating the cancerous from the non-cancerous melanomas.

STUDIES ON SPONTANEOUS NEOPLASMA IN FISHES. IV. GANGLIONEUROMA IN THE MARINE FISH, *HALICHOERES BIVITTATUS* (BLOCH). FROM BIMINI, B.W.I. Ross F. NIGRELLI. (New York Aquarium, New York Zoological Society, New York, N.Y.)

Ganglionerromatas in the Slippery Dick appear as nodules or raised wavy patches on the surface of the body. They vary in size from a few millimeters for the nodules to several centimeters for the patches. The growths are well vascularized and occasionally heavily pigmented, especially if they occur in regions that normally show a heavier concentration of melanophores (e.g., the lateral line). Histologically, the growth is composed of adult myelinated ganglion cells together with their processes and supported by a well developed reticulum. The hyperplasia extends from the eorial region into the epithelium and over the scales. There is a tendency to infiltrate the adjacent tissues and where the growth is found on the fins, the bony rays of these appendages are invaded and replaced by the tumor cells. No metastases, however, were noted. Attempts to transplant the tumors to other areas in the same fish, and in normal fish, were without success. Seventeen hundred and eighty-eight fish were examined and 1.57 per cent were found with one or more of these growths on the body. The incidence was greater among females than either males or immature fish.

EFFECT OF VASCULAR OCCLUSION ON TRANSPLANTED TUMORS. JULIUS S. YOUNGNER and GLENN H. ALGIRE. (National Cancer Institute, Bethesda 14, Md.)

Previous work of the authors has indicated the importance of host vascular reactions, probably including local tissue anoxia, in the mechanism of action of certain tumor-damaging substances. In the present study, mechanical interference with tumor blood supply was used to simulate changes in circulatory supply resulting from injection of the tumor-damaging substances previously studied.

Microscopic observations *in vitro* were made of the reactions of normal and growing neoplastic tissues included within transparent chambers in CSH mice, prior to and following mechanical occlusion of the blood supply to the window area.

In the case of control windows without tumor transplants, vascular occlusion for periods of from 1 to 3 hours resulted in no significant visible changes in any of the tissues under observation. Vascular levels returned to normal rapidly.

When the blood supply to windows bearing sarcoma transplants was obstructed for similar periods, hemorrhage developed in the tumors within 1 hour after flow was resumed and necrosis was observed 24 hours later. Normal tissues surrounding the sarcoma were unaffected. In all cases, renewed vascularization and growth of peripheral sarcoma cells occurred. In contrast, mammary carcinomas treated in the same manner were not affected, vascular levels rapidly returned to normal without any signs of hemorrhage or necrosis.

These findings are discussed in relation to: (a) importance of host circulatory reactions in tumor damage caused by certain substances; (b) differences in response of vascular networks of sarcomas and carcinomas under the conditions of the experiments reported.

THE EFFECTS OF SEVERAL AMINOAZO DYES ON THE INTRACELLULAR COMPOSITION OF RAT LIVER. J. M. PRICE (by invitation), E. C. MILLER, J. A. MILLER, and G. M. WEBER (by invitation). (McArdle Memorial Laboratory, University of Wisconsin, Madison 6, Wis.)

Homogenates of the livers of rats fed various aminoazo dyes for four weeks were separated by differential centrifugation into nuclear, large granule, small granule, and supernatant fluid fractions. The fractions and whole homogenates were analyzed for protein, nucleic acids, riboflavin, and protein-bound aminoazo dye. The data have been compared with earlier data (Price *et al.*, *J. Biol. Chem.*, **173**:345, 1948; *Cancer Research*, **9**, In press) on the same fractions from normal liver and from liver tumors induced by 4-dimethylaminoazobenzene. Dyes which are more or less carcinogenic than 4-dimethylaminoazobenzene produced greater or lesser changes, respectively, than 4-dimethyl aminoazobenzene. Thus, a carcinogenic dye (*e.g.*, 3'-methyl-4-dimethylaminoazobenzene) tended to make the liver more like tumor tissue while the non-carcinogenic 4-aminoazobenzene did not alter the composition of the liver. Increased contents of desoxypentosenucleic acid and protein in the nuclear fraction, decreased contents of protein, riboflavin, and pentosenucleic acid in the large granules, a decreased content of pentosenucleic acid in the small granules, and an increased pentosenucleic acid content in the supernatant fluid appeared to be characteristic of pre-neoplastic liver.

The non-carcinogenic dye 2-methyl-4-dimethylaminoazobenzene was a peculiar exception. It greatly increased the protein and riboflavin contents of the large granules. Thus the composition of this fraction changed in a direction opposite that found in tumor tissue. Decreases in the pentosenucleic acid contents of the large and small granules also occurred. As with the other dyes, protein-bound dye was formed in each fraction.

THE EFFECTS OF SEVERAL AMINOAZO DYES ON THE SUCCINOXIDASE OF RAT LIVER.

V. R. POTTER, J. M. PRICE (by invitation), E. C. MILLER, and J. A. MILLER. (McArdle Memorial Laboratory, University of Wisconsin, Madison 6, Wis.)

Homogenates of the same livers that were studied by Price *et al.* (see previous abstract) were assayed for the succinoxidase system and the oxalaeetic oxidase systems by techniques previously described. The amount of succinoxidase was roughly proportional to the amount of protein and of riboflavin in the mitochondria, in which the enzyme has been shown to be localized. The three constituents were decreased by the carcinogenic dye 3'-methyl-4-dimethylaminoazobenzene and greatly increased by the 2-methyl compound, which is non-carcinogenic. The amount of enzyme did not appear to correlate with any of the other cell constituents.

When rats that had been maintained on the basal diet were placed on the same diet minus protein, the succinoxidase of the livers dropped to approximately the hepatoma level, which was also attained in 4 weeks by feeding the most potent dye.

Since the mass of the mitochondria in terms of protein, riboflavin, and succinoxidase per gram of liver or nuclear material can vary as much as 300 per cent from one experimental condition to another, it appears that the processes of synthesis and destruction of these cytoplasmic particles can proceed at rates that differ from the rates of the corresponding processes in the nucleus or in the cell as a whole. These observations suggest that the particles and the cells may have different rates of multiplication.

FACTORS AFFECTING THE DESTRUCTION OF AZO DYES BY HEPATIC CELLS *IN VITRO*.

CHARLES J. KENSLER. (Department of Pharmacology, Cornell University Medical College, New York 21, N.Y.)

Earlier reports from this laboratory have shown that diet influences the ability of rat liver slices to destroy the hepatocarcinogen N,N-dimethyl-p-aminoazobenzene (DMB) and that this activity of liver decreases as their riboflavin concentration falls.

DMB and a series of five related compounds of varying carcinogenic activity have been fed to rats maintained on a casein-dextrose type diet and the riboflavin concentration in the liver and the ability of liver slices to destroy DMB have been determined. The rate at which hepatic cells destroy DMB and eleven related compounds *in vitro* has also been determined. Methylation of either aromatic ring decreased the rate of destruction. Methylation of the 2' position produced the greatest decrease. Methylation of the amino group did not greatly decrease the rate of destruction whereas ethylation did. The oral administration of all six azo compounds decreased the ability of liver cells to destroy DMB *in vitro* but the administration of 2-acetylaminofluorene did not. This effect is more closely related to their rate of destruction by liver *in vitro* than to their carcinogenic activity. Livers from fasted rats (39 to 48 hrs.) also showed decreased activity.

The determination of the rate of destruction of the primary and secondary amino compounds was complicated by the fact that 30 to 40 micrograms were lost even though the cells were killed by the immediate addition of alkali. This loss can be prevented by the reducing agents thioglycerol or thioglycolic acid or by keeping the slices cold throughout the homogenization until the benzene extraction of the dye is begun. Heating the tissue (5 min. boiling water) did not prevent the loss which has been found to be associated with the action of KOH on the heat coagulated fraction. In contrast the tertiary amino compounds are destroyed only in fortified homogenates which are inactivated by heat in agreement with the recent report of Mueller and Miller. Cytochrome c greatly inhibits the destruction of DMB in fortified homogenates.

THE CARCINOGENICITY OF CERTAIN FLUORO DERIVATIVES OF 4-DIMETHYLAMINO-

AZOBENZENE IN THE RAT. J. A. MILLER, R. W. SAPP (by invitation), and E. C. MILLER. (McArdle Memorial Laboratory, University of Wisconsin, Madison 6, Wis.)

The activities of several fluoro and trifluoromethyl derivatives of 4-dimethylaminoazobenzene as hepatic carcinogens for the rat were determined. The 3'- and 4'-fluoro derivatives were more active than 4-dimethylaminoazobenzene while the 2'-fluoro derivative was at least as active as the parent dye. In contrast the 2', 3', and 4'-trifluoromethyl derivatives of the dye were inactive even after feeding for 8 months. The high activities of the 2'- and 4'-fluoro compounds are of interest since previous work has shown that substitution in these positions with 5 other groups produced compounds with activities considerably below that of the parent compound. Furthermore, the presence of the very strong carbon-fluorine bond in the 2'- and 4'-positions of these active molecules makes it unlikely that a benzidine rearrangement of these dyes occurs *in vivo*. A recheck on the carcinogenicity of 2,4'-diamino-5-dimethylaminobiphenyl, the hypothetical benzidine rearrangement product that might be derived from 4-dimethylaminoazobenzene *in vitro*, showed again that this compound was inactive even after being fed at high levels for 8 months. Two tri-substituted derivatives, 2', 4', 6'-trichloro-, and 2', 4', 6'-tribromo-4-dimethylaminoazobenzene, were also inactive. These data and those previously reported on structural variations (Miller and Miller, *J. Exper. Med.*, 87:139, 1948) indicate that steric factors predominate amongst the several factors that probably govern the carcinogenicity of ring-substituted derivatives of 4-dimethylaminoazobenzene.

THE CARCINOGENIC ACTIVITY OF SEVERAL STRUCTURAL ANALOGS OF 2-ACTAMINOFLUORENE IN THE RAT. E. C. MILLER, J. A. MILLER, R. B. SANDIN (by invitation), and R. K. BROWN (by invitation). (McArdle Memorial Laboratory, University of Wisconsin, Madison 6, Wis., and Department of Chemistry, University of Alberta, Edmonton, Alberta, Canada.)

To study some structural features involved in the carcinogenic activity of 2-acetaminofluorene, 0.036 per cent of 2-acetaminofluorene and equimolar levels of 3-acetaminodibenzothiophene, 3-acetaminodibenzothiophene-5-oxide, and 3-acetaminodibenzofuran were fed in a grain diet for 8 months. Each compound induced mammary adenocarcinomas in female rats, but 2-acetaminofluorene and 3-acetaminodibenzothiophene were the most active; 60 per cent of the females fed these compounds had tumors at 4 months. Squamous cell carcinomas arising from or near the ear duct developed in rats fed each compound; the incidences were about 50 per cent for both the male and female rats fed 2-acetaminofluorene and 3-acetaminodibenzothiophene for 8 months. Only 2-acetaminofluorene elicited liver tumors; they were found in 60 per cent of the males fed this compound for 8 months. In another connection 0.105 per cent of 4-dimethylaminodiphenyl was fed in a purified diet for 8 months. Of the 10 males surviving for 8 months 3 developed subcutaneous tumors and 2 had squamous cell carcinomas arising from or near the ear duct.

These data demonstrate the effect of substituents in the central ring on the carcinogenicity of 2-acetamino-

fluorene. Replacing -Cl₂- by -S- does not alter the carcinogenicity of the molecule for either mammary or ear duct tissue but abolishes its activity for the liver.

Substitution of -S- or -O- diminishes the activity for the mammary and ear duct tissue and abolishes it for the liver. Omission of the -CH₂- bridge as in 4-dimethylaminodiphenyl still permits the induction of subcutaneous and ear duct tumors.

PRECANCEROUS CHANGES IN THE LIVER PROTEINS OF RATS FED ACETYLAMINOFLUORENE. A. CLARK GRIFFIN, HYLA COOK, and J. MURRAY LUCK (Introduced by JOSEPH C. AUN). (Department of Chemistry, Stanford University, Cal.)

Albino rats were maintained on purified diets containing 0.04 per cent acetylaminofluorene for a period sufficiently long to induce liver tumors. Animals were sacrificed at approximately one month intervals during this precancerous period and the total nitrogen, phosphorus, deoxyribonucleoprotein, ribonucleoprotein, and riboflavin content of the livers determined. Similar determinations were also made on liver tumors induced by this agent. Parallel observations were made on the serum proteins by the electrophoretic method.

During acetylaminofluorene carcinogenesis there was a decrease in the liver deoxyribonucleoprotein which is in contrast to the azo dye carcinogenesis wherein this fraction increases. The liver concentration of ribonucleoprotein, riboflavin, nitrogen, and phosphorus also decreased as the acetylaminofluorene was fed. Liver tumors resulting from this agent had lower concentrations of deoxyribonucleoprotein, nitrogen and phosphorus than liver tumors induced by the azo dyes. The livers showed a progressive increase in size as the diet containing the acetylaminofluorene was fed.

The dietary acetylaminofluorene resulted in some increase in the β -globulin of the serum, other serum proteins remained relatively normal. This is contrasted to the increase observed in the serum γ -globulin fraction and no apparent effect on the β -globulin component that results from the carcinogenic azo dyes.

HEPATOMAS FOLLOWING INTRAHEPATIC INJECTION OF "MITOCHONDRIA FRACTION" OF CHEMICALLY INDUCED HEPATOMAS. J. STASNEY, K. E. PASCHKIS, and A. CANTAROW. (Jefferson Medical College, Philadelphia 7, Pa.)

Portions of hepatomas induced in 37 rats by feeding 2-acetaminofluorene were homogenized and fractionated by the differential centrifugation technique of Claude, as modified by Hogeboom *et al.* A sample of each hepatoma "mitochondria fraction" was examined for the presence of intact cells or nuclei. None was found. One hundred gm. of normal liver was subjected to the same procedure and the entire "mitochondria fraction" examined for the presence of intact cells or nuclei. None was found.

Various fractions were injected into or implanted in the livers of normal adult rats under ether anesthesia.

Forty-six rats were thus inoculated with the "mitochondria fraction." Others were so inoculated with (a) crystals of acetaminofluorene suspended in plasma, (b) suspensions of intact hepatoma cells, and (c) the fraction removed at the first centrifugation, containing coarse cellular and nuclear particles and perhaps some intact nuclei.

The host animals were sacrificed at varying intervals following inoculation. Typical hepatomas were found, after 29 and 35 days, respectively, at the site of inoculation in 2 of 36 rats receiving the "mitochondrial fraction" that survived longer than 7 days. None was present in any of 48 animals receiving the other materials. Focal necrosis with fibroblastic reaction was frequently present at the inoculation site, especially in animals receiving the "mitochondria fraction." The significance of these observations is discussed.

TUMOR PRODUCTION AND METABOLISM OF AZOTOLUENE, AZOTOLUENE SPLIT PRODUCTS AND ANILINE IN RATS ON VARIOUS DIETS. B. EKMAN and J. P. STRÖMBECK (Introduced by KONRAD DOBRINGER). (Department of Biochemistry and University Clinie, University of Lund, Sweden)

Tumors of the bladder have been produced by feeding rats on a restricted diet described by Bowman and Miller together with 2,3-azotoluene or its split products o- and m-toluidine and o- and m-aminobenzoic acid. The same type of tumors are produced by feeding aniline on the same diet whereas p-aminophenol does not give any tumors. The metabolism of these compounds was studied in rats on various diets and it was observed that with a complete diet when no tumors were obtained, increased amounts of the oxidized excretion products (aminobenzoic acids, aminocresols and p-aminophenol) were found in the urine. It has been possible to influence both the metabolism of the tumorogenic compounds and the tumor frequency by varying the protein and vitamin B (especially riboflavin) content of the diet. The increased output of the oxidized compounds was parallel to an increased synthesis and excretion of ascorbic acid. The role of the ascorbic acid in the metabolism of the compounds studied will be discussed.

ANOMALOUS ACTION OF CROTON OIL AS CARCINOGENIC PROMOTING AGENT. I. BERENBLUM (by invitation). (National Cancer Institute, Bethesda 14, Md.)

Croton oil, itself non-carcinogenic, can elicit tumors in mouse's skin pre-treated a few times, or even once only, with a carcinogen (Berenblum, 1941, Mottram, 1944). Under such conditions, the total tumor yield is dependent on the effectiveness of the preliminary action of the carcinogen, while the average latent period is a function of the subsequent croton oil treatment. (Berenblum and Shubik, 1947; 1949, *In press.*)

A slight, but significant, difference in latent period was noted between the earlier and later series, both carried out in Oxford, England. The use of different batches of croton oil, in the two series, and the fact that mice

from different sources were employed, might both have been responsible for the differences observed.

A further investigation by the author is now in progress at the National Cancer Institute, Bethesda, employing croton oil of yet another source (from this country), and using 5 different inbred strains of mice (C, C₃H, dba, C57 brown, and C57 black). These experiments, not yet completed, already indicate a low promoting effect of croton oil (less than 1 per cent tumor yield after 10 weeks of croton oil treatment, as compared to over 30 per cent, after the same interval in previous studies). About 600 mice are being employed in this study.

Possible explanations of these results to be considered are: (a) variation in concentration of the 'promoting factor' in different batches of croton oil; (b) presence of anticarcinogenic substances in some batches of croton oil; (c) influence of strains of mice used; and (d) conditions of maintenance (diet) and housing of animals, etc. These will be discussed.

SOME OBSERVATIONS ON THE BIOCHEMICAL EFFECTS OF BERYLLIUM. ROBERT S. GRIER, MAHILON B. HOAGLAND, and MARGARET HOOD (Introduced by JOSEPH C. AUB). (From the Medical Laboratories of the Collis P. Huntington Memorial Hospital of Harvard University at the Massachusetts General Hospital, Boston, Mass.)

The relative simplicity of the element beryllium as a sarcomagenic and rachitogenic agent and its close periodic relation to magnesium warrant study of the biochemical phenomena involved. Because of the importance of alkaline phosphatase in bone metabolism and of the activating effect of magnesium thereon, the effect of beryllium on several phosphatases has been studied.

Beryllium in small concentrations markedly inhibits the alkaline phosphatase activity of crude extracts of bone, intestine, serum, and sarcoma tissue as well as a relatively purified intestinal phosphatase. Our observations suggest a competitive relationship between beryllium and magnesium to this enzyme. Furthermore, reversal of magnesium activation and beryllium inhibition can be produced by adding citrate to the system.

INDUCTION OF ADENOCARCINOMA AND OTHER LESIONS OF GLANDULAR STOMACH IN RATS BY INTRAMURAL INJECTION OF 20-METHYLCHOLANTHRENE. HAROLD L. STEWART, WILLIAM V. HARE (by invitation), EGON LORENZ, and JAMES G. BENNETT (by invitation). (National Cancer Institute, Bethesda 14, Md.)

Six-tenths milligram of methylcholanthrene suspended in an aqueous solution of methyl cellulose (Methocel 4000 cps.) was injected at each of two sites into the wall of the glandular stomach of rats of the Osborn-Mendel, Marshal 520, and AXB strains. Of 96 animals which have been autopsied the following lesions were observed: adenocarcinoma, 1; adenocanthoma, 2; sarcoma, 4; and a massive diverticulum like lesion with atypical proliferation of epithelium, 57. The morpho-

logic characteristics of this latter lesion, its pathogenesis, its possible neoplastic nature, and the results of transplantation are presented. A marked difference was noted in the number of lesions induced depending upon the site of injection, the prepyloric region being the more responsive.

BIOSYNTHESIS OF AMINO ACIDS UNIFORMLY

LABELED WITH RADIOACTIVE CARBON,
FOR USE IN THE STUDY OF GROWTH.
IVAN D. FRANTZ, JR. and HOWARD FEIGELMAN
(Introduced by PAUL C. ZAMECNIK). (From the
Medical Laboratories of the Collis P. Huntington
Memorial Hospital of Harvard University, at
Massachusetts General Hospital, Boston, Mass.)

The usefulness of amino acids labeled with radioactive carbon has been demonstrated in a number of laboratories. With a few notable exceptions, most of the experiments have been carried out with the simpler, non-essential amino acids, primarily because of the difficulty of synthesizing the more complicated molecules from the radioactive starting materials available, and the low yields obtainable. It appears that more significant results could be obtained with compounds which the tissues being studied are incapable of synthesizing.

The purpose of the present work was to produce useful quantities of the essential amino acids by biosynthesis. The autotrophic bacterium *Thiobacillus thiooxidans* was grown in an atmosphere of radioactive carbon dioxide. The bacterial proteins were isolated and hydrolyzed, and the amino acids separated by starch column chromatography. Because labeled carbon dioxide was the sole source of carbon, the amino acids were uniformly labeled in all positions. This point was confirmed by comparisons of the specific activities of the various amino acids with each other, with allowance for the number of carbon atoms in each. Sufficient quantities were obtained for further studies of tumors in animals. The specific activity of the products was about 150 microcuries per millimole of carbon. The culture vessel was so arranged that any carbon dioxide not utilized by the bacteria could be recovered.

A STUDY OF THE RELATIVE TOXICITY OF N-
IDEOACETYL AMINO ACIDS AND OF TISSUE
DISTRIBUTION OF RADIOACTIVE
ANALOGUES CONTAINING I^{131} IN RELA-
TION TO INHIBITION OF GROWTH OF
SARCOMA 37 IN SWISS MICE. ORRIE M.
FRIEDMAN and ALEXANDER M. RUTENBURG (In-
troduced by ARNOLD SELIGMAN). (Department of
Chemistry, Harvard University, Cambridge,
Mass., Kirsstein Laboratory of Surgical Research,
Beth Israel Hospital, Boston, and Department of
Surgery, Harvard Medical School, Boston, Mass.)

Since toxic substances related to essential metabolites seemed of interest for a study of inhibition of growth of tumors, derivatives of amino acids were prepared which were toxic and which could be readily labelled with a radioactive isotope. N-iodoacetyl derivatives of tryptophane, leucine and phenylalanine have been prepared. The relative toxicity in Swiss mice of

these 3 substances and iodoacetamide was determined and the ability of the 4 substances to inhibit the growth of Sarcoma 37 in this test animal was studied. The results have indicated that these substances inhibit the growth of this tumor significantly, to different extents and in a manner apparently unrelated to systemic toxicity.

The radioactive analogues of the three iodoacetyl amino acids and iodoacetamide have been prepared by the use of I^{131} . The concentration of radioactivity and its disappearance from blood, tumor, and liver following intravenous injection of these substances have been determined. Radioactivity has been found in the 3 tissues in significant amounts, the concentration of activity in tumor being consistently greater than in liver and less than in blood. The rate of disappearance of radioactivity from tumor in the case of the 3 amino acid derivatives followed a similar characteristic pattern different from that of iodoacetamide.

A LABILE CALCIUM-RIBONUCLEOPROTEIN
COMPLEX IN THE REGION OF THE LIVER
CELL CORTEX. T. B. ROSENTHAL and A. I.
LANSING (by invitation). (Department of Anatomy,
Washington University Medical School, St.
Louis, Mo.)

A calcium binding mechanism apparently associated with the cell surface undergoes characteristic changes with age and with some abnormal growth patterns (hyperplasia, cancer). We have suggested that this calcium binding mechanism is related to cellular growth. The experiments here reported add information on the chemical nature and localization of intracellular calcium binding.

By employing radiocalcium as a tracer, pyronin as a marker, and "Celite" as a selective adsorbent in a chromatographic procedure we have shown in homogenates of liver cells that calcium is bound to a nucleoprotein of the ribose type. By employing ribonuclease, both in the chromatographic column and in the cytochemical localization procedure of Brachet, further evidence has been found that the cell cortex is the region of calcium binding.

INITIAL USE OF HIGH ENERGY DEUTERONS
AND ALPHA PARTICLES IN CANCER RE-
SEARCH.* C. A. TOBIAS (by invitation), PAUL
ROSAHN, HAL ANGER (by invitation), and JOHN H.
LAWRENCE. (Division of Medical Physics, Radia-
tion Laboratory, and Department of Physics, Uni-
versity of California, Berkeley, Cal.)

Particle beams of high energy ions produced by accelerators, such as the 184" cyclotron in Berkeley(1), have several applications in the study of the biological effects caused by irradiation and of tumors. Measurements performed on 190 Mev deuterons and 380 Mev alpha particles indicate: (a) that such beams penetrate in tissue to a depth of approximately 14 and 7 em respectively, with the ionization distributed overwhelmingly in the main core of the beam, and negligibly in the

* This work has been carried out under the auspices of the A.E.C. See also AECD 2099-A.

fraction scattered out, or in the secondary neutrons produced; (b) the curve of specific ionization has a sharp peak, less than 1 cm from the end of the range. The ratio of peak ionization, to the ionization of the particles as they emerge from the cyclotron, is about 4 : 1 for deuterons and 6 : 1 for alpha particles. In agreement with calculations made by Wilson (2), such beams appear applicable to concentrated depth doses of irradiation. Most of the ionization may be concentrated in a sphere $\frac{1}{2}$ " diameter at any part of the body, while the ionization in the tissues not directly in the path of the beam is reduced to a minimum. The ratio of depth dose to surface dose, and the ability to direct the beam to a local spot, appear to be much superior to any other type of radiation. Some preliminary experiments were carried out by using type "A" mice and Strong transplantable mammary carcinoma. The deuteron beam was passed through the body of the animals with absorbers placed in the beam in such a way that the tumors received the highest specific ionization. Some of the tumors showed complete regression this way, without producing too severe radiation damage to the body of the animals. A more complete study is under way concerning specific physiological and biochemical effects produced by irradiation of the animal body, tumors and specific organs.

1. W. M. BROBECK, E. O. LAWRENCE, K. R. MCKENZIE, E. M. McMILLAN, R. SERBER, D. C. SEWELL, and R. L. THORNTON. The initial performance of the 184" Cyclotron of the University of California. *Physical Review*, 71:1947.
2. R. R. WILSON. Radiological Use of Fast Protons. *Radiology*, 47:487, 1946.

ANALYSIS OF LYMPHOMA INDUCTION BY X-RAY IN MICE. GEORGE A. SACHER (by invitation), and AUSTIN M. BRUES. (Argonne National Laboratory, Chicago, Ill.)

CF-1 female mice 120 days of age were treated with total body X-ray, using single and daily fractionated dosages between 400 and 1200 r. The incidence of lymphoma was determined (grossly at autopsy) and studied as a function of time and treatment. The total cumulative incidence is determined by the size of the dose and the fractionation pattern. When the logarithm of the rate of morbidity is plotted against time after treatment, a set of curves is obtained which is characterized by a peak in morbidity a few months after treatment, with a subsequent approach to the normally rising control morbidity curve. The incidence a year or more after treatment is little in excess of that of controls of the same age. When the morbidity rates are considered as the sum of the normal age incidence and the superimposed carcinogenic response, it is found that the carcinogenic response curve changes in amplitude (but not in form) with alterations in the dosage.

EFFECTS OF IRRADIATION WITH X-RAYS ON MAMMARY TUMOR TRANSPLANTS OBSERVED IN VIVO. RUTH M. MERWIN, GLENN H. ALGIRE, and HENRY S. KAPLAN. (National Cancer Institute, Bethesda 14, Md.)

Direct microscopic observations through a mica window have been made of the effects of X-radiation on mammary adenocarcinomas transplanted beneath the skin of strain C3H mice. The tumors were exposed to $2\frac{1}{2}$ to five thousand r, the remainder of the animal being shielded. At the time of treatment the implant had usually been in about a week and was 2 to 8 mm. in diameter. Effects were observed on tumor growth rate, vascular reactions, translucency, appearance of fat cells and acinar structures.

Six hours after irradiation the only reaction noted is enlargement of the tumor. Eighteen to thirty hours after treatment no further increase in size is found although there is sometimes shrinkage from the original size at the higher doses. A general opacity of the tumor tissue is conspicuous but no evidence of vascular damage is observed. During the next few days the tumor decreases in size and the vascularity is greatly reduced as the vessels become much narrower or disappear. Resumption of growth of tumor tissue after irradiation was followed in two instances. The first indication of recovery was the enlargement of blood vessels in scattered areas, followed by the development of translucency and the progressive growth of these foci.

The interpretation of the observed changes is discussed in relation to the role of the vascular changes following irradiation.

HISTOLOGICAL CHANGES PRODUCED BY A SINGLE LARGE INJECTION OF RADIO-PHOSPHORUS (P^{32}) IN ALBINO RATS AND IN C3H MICE. B. GRAD and C. E. STEVENS (Introduced by C. P. LEBLOND). (Department of Anatomy, McGill University, Montreal, Canada)

Young male and female rats and adult female C3H mice with spontaneous mammary tumors received a single large dose of radio-phosphorus and were sacrificed 2 hours to 10 days later. The destructive effects of P^{32} are most marked in lymphatic organs and bone marrow, small intestine, granulosa of ovarian follicles, and mammary tumors. Minor changes are noted in stomach and colon and at later intervals in the seminiferous tubules of testis, and hair follicles. The cellular destructions observed in all these organs consist of cloudy swelling and nuclear pyknosis. Nuclear abnormalities such as gigantism are found in recovering cells. In the crypts of the small intestine, massive degeneration and pyknosis of dividing cells results in thinning of the epithelium of crypts and villi, probably because no new cells are supplied from the crypts to balance the continual loss of cells by extrusion normally occurring at villi tips.

The mammary tumors show many scattered pyknotic cells at 24 hours after injection. At 48 hours large blood lakes have formed without much tissue damage. At 10 days most of the tumor is necrotic and liquefied probably because of interference with the blood supply. Little or no damage is observed in salivary glands, pancreas, liver, genitourinary system, endocrine glands, nervous, muscular, and connective tissue.

Thus, as with X-radiation, the major changes occur in organs with a rapid cell turnover. These organs were previously shown to take up large amounts of radio-phosphorus from the blood.

EFFECTS OF CHRONIC IRRADIATION WITH GAMMA RAYS ON MAMMARY TUMOR INCIDENCE IN C₃H₂ FEMALE MICE. EGON LORENZ, WALTER E. HESTON, and ALLEN B. ESCHENBRENNER. (National Cancer Institute, Bethesda, Md.)

One month old C₃H₂ (C₃H without the milk agent) female mice were exposed to 5.5 r of gamma radiation for 8 hours daily and autopsied when in moribund condition. The maximum accumulated dose was approximately 5900 r. Mammary carcinomas were observed in 23 per cent of the animals with a mean tumor age of 15 months and sarcomas at the site of the mammary glands were found in 33 per cent with a mean tumor age of 16 months. Comparing the experimental data on mammary tumor incidence with those of Heston ('1) on extensively bred C₃H₂ mice, the conclusion can be drawn that irradiation hastens the appearance of mammary carcinomas without increasing the incidence above that following extensive breeding. While sarcomas at the site of the mammary gland are rare in breeders, a high incidence of this tumor was found in the irradiated animals. The possible connection of this tumor with granulosa cell type tumor of the ovaries will be discussed.

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LOCAL IRRADIATION AND THE INDUCTION OF LYMPHOID TUMORS IN MICE. HERBERT S. KAPLAN. (Department of Radiology, Stanford University School of Medicine, San Francisco, Cal.)

Whole-body exposure of mice to roentgen rays in adequate dosage has long been known to yield lymphoid tumors. Strain A mice, which are moderately susceptible and strain C₅₇ black mice, which are highly susceptible to whole-body irradiation, have been studied following local exposure. Strain C₅₇ black mice one month of age were treated over the anterior or posterior half of the body with daily doses of 100 r for 10 days. After 17 months, only 2 of 55 mice (4 per cent) irradiated over the upper half, and 1 of 55 mice (2 per cent) irradiated over the lower half of the body have developed lymphoid tumors. In previous experiments the incidence of such tumors following whole-body exposure of young mice of this strain to the same total dose has averaged about 65 per cent at 10 months. No exact data are available for half of this total dose delivered to the whole body, but extrapolation indicates that a significantly higher incidence might be expected than was observed after half-body irradiation.

Larger doses sharply localized to the region of the mediastinum or upper abdomen have produced no lymphoid tumors in either strain, and mice of strain A

have also been completely refractory to local treatment over the skull or lower abdomen. These results suggest that shielding of one-half or more of the body from roentgen rays confers a considerable degree of protection against the induction of lymphoid tumors in mice.

TYPES OF TRANSPLANTABLE OVARIAN NEOPLASMS INDUCED BY X-RAYS AND THEIR PATHOGENESIS. T. BALI (by invitation), and J. FERTE. (Veterans Administration Hospital, Southwestern Medical College, Dallas, Texas)

Most mice exposed to a single 50 r or more develop ovarian growths. Exposure of ovaries seems essential; irradiation of pituitary region is neither productive nor inhibitory. Prenatal irradiation did not yield ovarian tumors, while irradiation at 1 to 3 days of life did, in most animals. Most mice exposed to 150 r at this age are sterilized, but even those having multiple, seemingly normal pregnancies, eventually developed ovarian tumors. The primary growths are usually complex: granulosa, luteoma, and rarely, angioma and sarcoma-like. Practically all of these are benign, e.g., when killed at 19 to 23 months of age, they were found in the majority of mice of the series irradiated at 1 to 3 days of age.

The common tubular structures were least transplantable in series. Those successfully grafted were exceedingly slow-growing.

The x-ray induced growths seem to possess a greater autonomy than those induced by the Biskind procedure. Many of them appear mere massive hyperplasias. Only granulosa tumors and luteomas secrete hormones, and their mother cells are known to be under hormonal control; the mother cell of the common tubular adenomas and the rare types of neoplasms mentioned are neither hormone-producing, nor under hormonal control.

Several factors can be implicated in the genesis of these tumors: a. Excessive pituitary gonadotrophic stimulation as indicated by the Biskind procedure; b. X-rays seem to exert a specific neoplastic stimulus, as on skin and other organs, non returning to "normal" once irradiated; c. Some local derangement of interdependent structures.

FREE AMINO ACIDS IN NORMAL AND NEOPLASTIC TISSUES AS STUDIED BY PAPER CHROMATOGRAPHY. EUGENE ROBERTS. (Department of Anatomy, Division of Cancer Research, Washington University Medical School, St. Louis, Mo.)

A survey was made of the free amino acids found in alcoholic extracts of freshly excised normal and neoplastic tissues. Two-dimensional chromatograms were made on aliquots corresponding to 75 mg. of fresh tissue after oxidation with H₂O₂ on the paper. Sixteen different normal tissues and samples of squamous cell and mammary carcinomata and sarcoma 37 have been examined. Epidermis showed the highest concentration of free amino acids of any of the tissues studied, a finding consistent with the extremely high level of trichloroacetic acid soluble nitrogen. With the exception of epidermis, the malignant tissues studied had greater overall concentrations of detectable constituents than did the nor-

mal tissues. The chief ninhydrin-reactive substances detected in normal tissues were glutamic acid, aspartic acid, glycine, taurine, alanine, serine, glutamine, cysteine, valine, and the leucines. Histidine was present in large quantities in brain, in small quantities in epidermis and tumors, and was not detectable in the other tissues. Arginine and lysine were found in appreciable quantities only in extracts of epidermis, while proline and hydroxyproline were detected in the epidermis and tumors, but not in other tissues. Some of the interesting differences observed among normal tissues will be discussed. Sample chromatograms will be shown.

There was a consistent pattern of distribution of free amino acids in the malignant tissues which was different from that of any of the normal tissues studied.

THE RELATIVE METABOLISM IN VITRO OF ANALOGOUS MOUSE MAMMARY TUMORS.

ANNA GOLDFEDER. (Cancer Research Laboratory, Department of Hospitals, City of New York, and Department of Biology, New York University, New York, N.Y.)

In a previous report ("Growth in Tissue Culture of Analogous Mammary Carcinomas and Their Response to Radiation," presented at the 1948 meeting of the American Association for Cancer Research—in press, *Cancer Research*), it was demonstrated that two analogous mammary tumors autogenous to homozygous hosts of dba and C3H strains of mice, both histologically diagnosed as adenocarcinomas, differed widely in regard to their rate of growth and radiosensitivity.

The present study is concerned with the relative rates of respiration, aerobic and anaerobic glycolysis of the above mentioned tumors.

Actively growing tumors, free from necrotic portions, were used. For the determination of respiratory changes the Barcroft-Warburg manometric technique was employed; for the determination of lactic acid in the substrate the Friedman-Contonio-Wendel method was used. Results obtained showed the metabolism of these two tumors (dba, C3H) to differ significantly. Thus Q_{O_2} of the mammary tumor of the dba strain averaged 5.6; Q_{CO_2} averaged 25.2; Q_{lact} averaged about 75.0 (dry weight, 60 minute basis), while the Q_{O_2} of the C3H mammary tumor averaged 3.8; Q_{CO_2} 8.6; Q_{lact} 55.8.

From both sets of experiments (growth and metabolism of the tumors used in this study) it is noted that, although both tumors appear histologically identical, nevertheless their biological and physiological characteristics appear to differ widely. One may infer that the morphological appearance of tumors is not a sufficient basis for their classification.

The significance of the metabolic rate in relation to radiosensitivity of tumors will be discussed.

YOLK SAC CULTIVATED TUMOR TISSUE AND EXPERIMENTS IN TUMOR CHEMOTHERAPY. II. NEUTRAL RED, ETHYL VIOLET, AND JANUS GREEN. ALFRED TAYLOR and IRVING GALINSKY (by invitation). (Biochemical Institute of the University of Texas and the Clayton Foundation for Research, Austin, Texas.)

Cancer tissue cultivated in eggs by the yolk sac method has been found to serve for the rapid testing of possible anti-cancer compounds. Eggs were inoculated with tumor tissue on about the fourth day of incubation. By the twelfth day tumor tissue was well established on the inner wall of the yolk sac. The test compound was injected on the twelfth day of incubation through a tiny perforation in the shell in such manner that the material was deposited between the shell and the chorio-allantoic membranes. The procedure did not increase the egg mortality over that of untreated controls. The experimental eggs and their saline injected controls were harvested 48 hours later on the fourteenth day of incubation and the effect of the test compound evaluated as it affected the weight of the tumor and the chick embryo. The yolk sac tumors were less variable in size and grew much more rapidly than the same tumor cultivated in its normal host, the C3H mouse.

Tests were made with neutral red, ethyl violet and Janus green which have been reported to inhibit cancer growth in mice. Altogether 11 experiments involving more than 300 tumor bearing eggs were completed. Neutral red at the maximum sublethal dose did not affect tumor or embryo growth. Ethyl violet inhibited tumor growth an average of 20 per cent; embryo growth was unaffected. Janus green slightly inhibited the growth of both tumor and embryo.

EFFECT OF FOLIC ACID ANTAGONISTS ON TRANSPLANTED MOUSE LEUKEMIA. ARTHUR KIRSCHBAUM, SISTER TERESITA JUDI (by invitation), NANCY GEISSE (by invitation), and LEO M. MEYER (Department of Anatomy, University of Minnesota Medical School, Minneapolis, Minn., and Department of Therapeutics, New York University Medical School, New York, N.Y.)

The effect of 9 folie acid antagonists (including amiopterin), teropterin, and folie acid on the survival time in 2 lines of myeloid and one line of lymphoid (?) transplanted leukemia was determined. The compounds were administered in maximum tolerable daily doses or doses comparable to those used clinically; treatment was begun on the day following transfer of the disease. Animals of the same transfer generations which received (a) either no treatment, or (b) Fowler's solution, or (c) urethane served as controls. The lines of leukemia originated in the F strain. Routine transfer was accomplished by intraperitoneal injection of one to five million splenic cells suspended in isotonic saline.

Survival was consistently but only slightly prolonged (compared to untreated mice) by the administration of the folic acid antagonists in the 2 lines of myeloid leukemia studied; in none of 90 was the development of the disease completely inhibited. Twenty-four of 36 animals receiving Fowler's solution (0.1 mg. daily) did not become leukemic, and in the others survival was longer than in mice receiving folie acid antagonists, teropterin, or folie acid. Urethane-treated controls also survived longer than test animals. In the third line where the cells were undifferentiated, and the type of leukemia may be open to question, and in 2 additional transfer lines of myeloid leukemia, similar results were

obtained. Neither folic acid nor teropterin accelerated the development of the transplanted leukemias.

THE EFFECT OF REGENERATION ON THE RATE OF PROTEIN SYNTHESIS AND DEGRADATION IN RAT LIVER. NANCY L. R. BUCHER and ROBERT B. LOFTFIELD, and IVAN D. FRANTZ, JR. (by invitation). (Medical Laboratories of the Collis P. Huntington Memorial Hospital of Harvard University at the Massachusetts General Hospital, Boston, Mass.)

The capacity of tissue to grow is intimately related to its ability to build proteins. A constant turnover of protein occurs under resting conditions. When additional protein is formed there must be either increased rate of synthesis, decreased rate of degradation, or both.

Using amino acids labeled with C^{14} we have investigated protein turnover in regenerating rat liver, a normal tissue that grows faster than most neoplasms. *In vitro* studies showed that slices of regenerating liver incorporated tagged alanine into protein at a maximum of 3.5 times the normal rate. *In vivo* experiments, using both alanine and glycine, demonstrated an increase of 1.5 to 2 times normal.

In degradation studies radioactivity was established in liver protein by administering labeled glycine. Inactive glycine was then force-fed to minimize the re-utilization of tagged molecules derived from breakdown products in the metabolic pool. The half-life of activity in the protein of resting liver was 3 days, whereas in non-glycine-fed rats a value of 5.5 days was obtained.

During growth, allowance must be made for dilution of tagged protein by new protein added. Regenerating liver provided a unique opportunity to determine this, since the amount of tissue left behind following partial hepatectomy could readily be calculated. When glycine was force-fed, the protein of regenerating liver was found to have a half-life of 5.5 to 11.5 days; without glycine radioactivity diminished very slowly.

These findings suggest that the rate of protein synthesis increases and possibly that of protein degradation decreases during hepatic regeneration.

THE INFLUENCE OF NORMAL SERUM AND HODGKIN'S SERUM ON CELLULAR GROWTH AND MORPHOLOGY IN TISSUE CULTURE. MARGARET S. REIMAN (by invitation), and HERMAN A. HOSTER. (The Ohio State University College of Medicine, Columbus, Ohio)

A study of the effect of normal serum and Hodgkin's serum on normal and diseased cells in tissue culture has been undertaken. An attempt has been made to compare the morphologic changes, which occur in normal cells and diseased cells as a result of the presence of Hodgkin's serum in the nutrient milieu, with the morphologic characteristics observed in the 3 types of Hodgkin's explant growth in the presence of normal serum.

The present study is based on 55 tissue specimens and 1500 explants cultured in the roller tube without transplantation for 3 to 20 weeks (average 6 weeks). All ob-

servations reported were made on living cells at 100 and 500 magnifications. Alterations in morphology and cellular growth patterns observed in cells nourished with Hodgkin's serum occurred following the use of most but not all sera samples studied.

Three general types of growth were observed in Hodgkin's lymph node cultures. These have been tentatively classified as follows: (a) the grannule-containing macrophage and/or reticulum cell type, (b) the granule-free syncytial round or elongated cell type, and (c) the fibroblast type. Alterations in the morphology and cellular growth pattern observed both in cells nourished with Hodgkin's serum and in Hodgkin's tissue growth types a, b, and c will be illustrated.

DIET AND AZO DYE TUMORS: EFFECT OF DIET DURING PERIODS WHEN THE DYE IS NOT FED. C. C. CLAYTON (by invitation), and C. A. BAUMANN. (Department of Biochemistry, University of Wisconsin, Madison 6, Wis.)

Rats fed m' methyl p-dimethylaminoazobenzene (m'DAB) develop hepatic tumors even though the feeding of the dye is interrupted for periods of 4 to 12 weeks. In a typical experiment the dye is incorporated into a standard synthetic ration and fed for 4 weeks, one of several dye-free diets is fed during the next 4 weeks, the feeding of the dye in the synthetic ration is repeated for 4 weeks, and during the final 8 weeks the dye-free synthetic ration is fed. Under this arrangement the number of tumors formed depends upon the diet fed during the period of interruption.

Diets high in fat or in methionine failed to alter tumor formation. Calorie restriction during the period of interruption not only failed to diminish tumor incidence, but actually appeared to enhance it. Diets high in riboflavin and protein decreased the number of tumors formed; while a diet low in labile methyl groups and containing nicotinamide increased the numbers of tumors. The results suggest that some of the so-called effects of diet on tumor formation may be exerted on the animal itself rather than on an altered metabolism of carcinogen.

In contrast to the effects of diet observed during the period of interruption no very pronounced effects of diet were noted when the carcinogen was fed continuously for 8 weeks and the dietary variation introduced subsequently.

BLOOD PRESSURE MEASUREMENTS IN TRANSPLANTED TUMORS OF UNANESTHETIZED MICE. GLENN H. ALGIRE. (National Cancer Institute, Bethesda, Md.)

The transparent chamber technique as adapted to a skin flap in mice makes accessible for microscopic observation a layer of subcutaneous and muscular tissue approximately 0.5 mm. thick, and having a surface area of 150 sq. mm. Microscopic observations of normal and neoplastic tissue transplants within the chamber may be made at magnifications up to 500 X.

An indirect method has been devised for blood pressure measurements of any vascular component within

the chamber during direct microscopic examination by transmitted light. Measurements may be made on the same vessel intermittently throughout the day, and repeatedly for the duration of the preparation (approximately 30 days). The apparatus consists of a mercury sphygmomanometer system and air reservoir connected to a glass tube having a flexible, translucent membrane tied loosely across the end. A micromanipulator is used to bring the membrane into contact with the undersurface of the skin. Pressure applied using the sphygmomanometer bulb results in slight bulging of the membrane. As the entire field is visualized under the microscope one can obtain a measure of the arterial, systolic and diastolic pressures, and venous pressures. The pulse wave can be seen at maximum amplitude approximately midway between systolic and diastolic pressures.

Parallel observations and measurements have been made in vessels of both normal and neoplastic tissues of such correlated vascular phenomena as changes in caliber of vessels, rates of flow, vasomotion, intravascular agglutination. Data are presented showing relationships of host and tumor blood pressure and vascular supply under various experimental conditions leading to tumor damage.

VIRAL HEPATITIS AND HODGKIN'S DISEASE.

HERMAN A. HOSTER and ROBERT P. ZANES, JR.
(by invitation). (The Ohio State University College of Medicine, Columbus, Ohio.)

The present study is concerned with the effect of an infectious disease, viral hepatitis, on the course of Hodgkin's disease. Reports dealing with the favorable influence of viral hepatitis on other disease entities have been limited to rheumatoid arthritis (1). More recent accounts have ascribed this phenomenon to "non-specific disturbances of liver function" and not to the presence of an infectious agent.

The material presented is based on two cases observed for 5 and 6 years following the onset of hepatitis, one case which terminated fatally with the onset of jaundice, and 21 cases observed for 3 to 8 months after inoculation with viral hepatitis containing sera and tissue extracts.

The evaluation of results presented is based principally on changes in hematologic equilibrium and on alterations in the clinical status of the patient. Unusual gross and microscopic findings in the single death resulting from hepatitis will be presented.

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MUCOPOLYSACCHARIDES AND MUCOLYTIC ENZYMES *IN VITRO*. NORMA E. SHIFRIN (by invitation), and WILLIAM L. SIMPSON. (Detroit Institute of Cancer Research, Detroit 1, Mich.)

Conflicting data on the occurrence of mucolytic enzymes in extracts of malignant tumors and in the effects of such enzymes on the invasive properties of malignant cells indicate the need for new and more carefully controlled studies on the relationship of these substances to malignancy. Attempts have been made to study these complex polysaccharides and related enzymes by tissue culture methods.

Preliminary experiments have been carried out to determine whether cells in culture can utilize or can acquire the ability to utilize mucopolysaccharides. The effect of varying concentrations of purified hyaluronic acid (HA) was tested on hanging-drop cultures made up of 1 drop of chicken plasma and 1 drop of 1 HA : 1 chick embryo extract were prepared to contain concentrations of 0.210 mg., 0.072 mg., and 0.021 mg. per culture.

In 7 out of 10 experiments using the above concentrations, growth inhibition seemed to be proportional to the amount of HA present. That is, the most growth occurred in the control groups, the least growth in the 0.210 mg. concentration groups. All of the cultures raised on concentrations of 0.210 mg. and 0.072 mg. died within 3 to 5 days after the last experiment. The cultures growing in a concentration of 0.021 mg. survived for as long as did the controls. The 10 experiments were carried out in 22 days. Attempts to repeat and extend these studies using the roller tube method of tissue culture are now being carried on.

A NEW REAGENT FOR THE HISTOCHEMICAL DEMONSTRATION OF ACTIVE CARBONYL GROUPS. ARNOLD M. SELIGMAN and RIVKA ASHBEL (by invitation). (Department of Surgery, Beth Israel Hospital, Boston, and Harvard Medical School, Boston, Mass.)

A hydrazide containing a naphthol nucleus has been synthesized. After the hydrazide has reacted with active carbonyl groups the naphthol moiety can be coupled with diazonium compounds to form colored pigments. Utilizing this reagent ketosteroid in adrenal cortex was converted to a deep blue pigment. Cells in the corpus luteum of ovary contain sufficient progesterone to give a deep blue pigment. *In vitro* tests showed that the reagent reacts with 3, 17, and 20 keto groups of steroid but not with 11-keto steroid. It may prove useful in urine analysis. The reagent may also be used for demonstrating aldehyde produced in collagenous tissue with periodic acid.

A rich source of carbonyl groups has been found in the non-lipoid component of the white matter of the central nervous system and peripheral nerves.

DISTRIBUTION STUDIES WITH NITROGEN MUSTARD CONTAINING RADIOACTIVE IODINE. ARNOLD M. SELIGMAN and ALEXANDER M. RUTENBURG, and ORRIE M. FRIEDMAN (by invitation). (Department of Surgery, Beth Israel Hospital, Boston, and Harvard Medical School, Boston, Mass.)

Diethyl (β -iodoethyl) amine hydrochloride and methyl bis-(β -iodoethyl) amine hydrochloride have been prepared with radioactive iodine (I^{131}). These compounds are a little more toxic than their β -chloroethyl homologues. Following intravenous injection in mice, rats, and humans, the rate of disappearance of radioactivity from the blood was similar to the rate of disappearance of radioactivity following the injection of an equivalent quantity of radioactive sodium iodide in the first 4 to 8 hours. After this period, a small quan-

ity of radioactivity persisted in the blood in the case of the mustards. Tissue analysis showed greatest persistence of radioactivity in lung, blood, and lymphoid tissue. Tumors contained no more radioactivity than other tissues. Following injection of the mustards ionic halogen is rapidly liberated in the blood. Only a few percent of the mustard reaches the tissues (except lung and blood) in uncyclized form.

FURTHER STUDIES ON THE METABOLISM OF 4-DIMETHYLAMINOAZOBENZENE BY RAT LIVER HOMOGENATE. G. C. MUELLER and J. A. MILLER. (McArdle Memorial Laboratory, University of Wisconsin, Madison 6, Wis.)

Rat liver homogenates fortified with diphosphopyridine nucleotide, nicotinamide, magnesium ion, and hexose diphosphate metabolized the hepatic carcinogen 4-dimethylaminoazobenzene in several ways. Thus small quantities of the demethylated derivatives 4-monomethylaminoazobenzene and 4-aminoazobenzene and a new metabolite, 4'-hydroxy-4-dimethylaminoazobenzene, were isolated from the reaction mixture. However, more dye disappeared from the reaction mixture than could be accounted for by the azo metabolites found. Presumably cleavage at the azo linkage occurred. The enzymes involved in these reactions appeared to be concentrated largely in the microsome and soluble protein fractions of the liver.

An additional factor involved in the metabolism of the dye was discovered when it was found that the rat liver homogenates lost the ability to metabolize the dye when incubated at 37° C. for 5 minutes. Fortification in the manner described above did not restore the activity but the preparation was reactivated when a liver koch-saft or a concentrate of triphosphopyridine nucleotide (TPN) was added. The probable identity of this factor with TPN was indicated by the observation that the reactivation of the incubated homogenates by the TPN concentrates was proportional to the content of TPN in these concentrates.

PRELIMINARY SCREENING OF 1000 CHEMICAL AGENTS FOR POTENCY IN PRODUCING DAMAGE IN SARCOMA 37. M. J. SHEAR and V. DOWNING, and J. L. HARTWELL (by invitation), J. LEITER, R. C. MACCAROLE, and A. PERRAULT and D. L. VIVIAN (by invitation). (Chemotherapy Section, National Cancer Institute, Bethesda, Md.)

More than 1,000 chemical agents have been screened against Sarcoma 37 *in vitro* in a preliminary fashion, viz.: a single maximum tolerated dose of each compound was injected subcutaneously into mice with week old implants of tumor; the animals were sacrificed at 8, 24, and 48 hours after injection. Gross and microscopic observations were made. The criteria employed in estimating damage induced in the tumors were the same as described in previous years.

In addition to the results presented in the accom-

panying abstracts, data obtained for other classes of compounds were as follows:

Chemical class	No. of compounds examined	No. yielding positive results
Quaternary ammonium salts	283	13
Acridines	41	7
Phenazines	30	3
Sulfonamides	12	0
Unsaturated ketones	34	3
Isoquinolines	17	0
Quinones	30	8
Stilbenes	17	5
Alkaloids	60	4
Di- and tri-phenyl methanes	40	2
Amidines and guanidines	24	1
Phenyl-C-C-C-phenyl ↓ NN	25	0

EFFECT OF PELTATINS ON CYTOCHROME OXIDASE ACTIVITY OF MOUSE TUMORS AND ORGANS. V. S. WARAVDEKAR (by invitation), and J. LEITER. (Chemotherapy Section, National Cancer Institute, Bethesda, Md.)

The cytochrome oxidase activity of 6 day old implants of Sarcoma 37 was determined in tumor homogenates obtained from mice sacrificed 1, 2, 4, and 8 hours after a single subcutaneous injection of 20 micrograms of alpha- or beta-peletatin per gram of body weight. A rapid reduction of enzyme activity to about 40 per cent of the activity of tumor homogenates from untreated animals occurred within 8 hours after injection. This effect paralleled closely the tumor damage seen histologically (MacCardle) in the same tumors. Injection of 500 to 1000 micrograms per gram of an inactive diastereoisomer and of an acetylated derivative of alpha-peletatin produced reductions in enzyme activity which corresponded roughly to the cytotoxic activity of these derivatives as observed histologically in adjacent portions of the same tumors.

The enzyme activity of liver, spleen, and kidney from the same animals showed little or no difference from the activity of organs from untreated tumor bearing control mice. Even lethal doses of 100 micrograms of the peltatins in mice not bearing tumors produced only slight lowering of the enzymic activity in these organs.

In experiments in which homogenates of Sarcoma 37, liver and spleen were incubated *in vitro* with the peltatins, only slight reduction of cytochrome oxidase activity was observed.

One hundred mg. per kilogram doses of the alpha-peletatin injected intravenously in rabbits likewise did not give a pronounced lowering of enzymic activity in liver and kidney.

ACTION ON SARCOMA 37 OF COMPOUNDS RELATED TO PODOPHYLLOTOXIN AND TO ALPHA- AND BETA-PELTATIN. J. LEITER and J. L. HARTWELL (by invitation). (Chemotherapy Section, National Cancer Institute, Bethesda, Md.)

Alpha- and beta-peletatin were converted into their diastereoisomers by treatment with alkali. The biologi-

eal activity, both as regards acute toxicity and effect on Sarcoma 37, following subcutaneous injection, was greatly reduced by this treatment. Doses of the new isomers up to 1000 micrograms per gram of body weight produced no deaths in mice and induced no effects in the tumors other than occasional mitotic arrest. In contrast, the parent substances produced grossly visible damage in tumors of various types at doses of two to five micrograms per gram of body weight.

Acetates, methyl and ethyl ethers of the peltatins and their diastereoisomers were prepared; the diastereoisomers and their derivatives had low biological activity whereas the peltatins and their analogous derivatives had high activity. The acetylated derivatives of podophyllotoxin and of its relatively inactive isomer pieropodophyllin had activities similar to those of the compounds from which they were derived.

Several benzoic acid derivatives, which represent oxidation residues of podophyllotoxin and of the peltatins, were found inactive against Sarcoma 37. A reduction product of podophyllotoxin was likewise found to be inactive under the conditions of these experiments.

A number of compounds structurally related to podophyllotoxin showed little or no activity at doses 500 times the minimum effective dose of podophyllotoxin. Among these were: conidendrin, dimethyl and diacetyl conidendrin, iso-olivil, isolariciresinol, hinokinin, mataresinol and its methyl ether, asarinin, guaiacetic acid, nordihydroguaiacetic acid, lariciresinol, sodium pinoresinol and its methyl ether, and sesamin.

TOXICITY AND HEMATOLOGIC CHANGES PRODUCED BY ALPHA-PELTATIN, BETA-PELTATIN AND PODOPHYLLOTOXIN. EZRA M. GREENSPAN (by invitation), and J. LEITER. (Chemotherapy Section, National Cancer Institute, Bethesda, Md.)

The maximum tolerated single dose (MTD) of podophyllotoxin, alpha-peltatin, and beta-peltatin in normal mice was approximately 30, 40, and 50 micrograms/gram, respectively. A single dose of 5 micrograms/gram regularly induced damage in several different types of transplanted mouse tumors. No gross toxicity or weight loss was observed in non-tumor bearing mice after repeated daily injections, for several weeks, of 5 micrograms/gram of alpha- or beta-peltatin.

The MTD for a single injection in rats without tumors was 5 and 10 micrograms/gram for alpha- and beta-peltatin, respectively. In rabbits the MTD for alpha- was 5 mg./kg., for beta-, >20 mg./kg. In dogs the maximum dose tolerated was 0.5-1.0 mg./kg. for alpha-peltatin, 1-2 mg./kg. for podophyllotoxin, and 2-4 mg./kg. for the beta-compound.

The subcutaneous, intramuscular, and intravenous routes of administration were used in mice, rats, and rabbits; dogs received the drug intravenously. Pronounced differences in MTD were not noted among the several routes of administration.

After parenteral injection of lethal or sublethal doses, toxic phenomena were observed in 2 to 6 hours. These included: diarrhea, salivation and emesis (dogs), respi-

ratory and central nervous system depression, muscular paralysis, and a definite pattern of response in the formed elements of the peripheral blood and bone marrow. No evidence of renal or hepatic toxicity was observed at these dose levels. Leucopenia developed $\frac{1}{2}$ to 2 hours after injection. This was followed by leucocytosis (with degenerating neutrophiles and lymphocytes), normoblastosis, and a terminal leucopenia 24 to 48 hours after injection. Degeneration and aplasia of bone marrow were observed at lethal doses.

RELATIVE TOXICITY TO NORMAL AND TUMOR BEARING MICE OF CERTAIN AROMATIC TRIVALENT ARSENICALS WHICH INDUCE HISTOLOGICAL DAMAGE IN SARCOMA 37. LYLE V. BECK, and ADRIAN PERRAULT, and JOYCE GILLESPIE (by invitation). (Chemotherapy Section, National Cancer Institute, Bethesda, Md.)

Of 39 pentavalent arsenicals tested (both aliphatic and aromatic), only sodium cacodylate produced gross and histological damage to Sarcoma 37 at maximum tolerated doses (MTD). On the other hand, of 24 trivalent aromatic arsenicals tested at MTD, or less, 6 produced gross and histological damage, and 5 others produced early cell damage and/or aberrant mitotic figures.

Certain of these trivalent arsenicals have been found to be less toxic to CAF₁ mice bearing week-old implants of Sarcoma 37 than to normal CAF₁ mice of the same sex, age, and body weight. For example, 3-amino-4-hydroxyphenyl dichlorarsine hydrochloride (Clorarsen) was found to have a 48 hour LD₅₀ value of $11.4 \pm S.E. 0.55$ micrograms of arsenic per gram in normal CAF₁ female mice, whereas the LD₅₀ value for such female mice with 6 day tumors was 16.9 ± 0.9 micrograms of arsenic per gram. Similar results were obtained in two additional experiments with this same compound, and in one or more experiments with the following: 3-acetamino-4-hydroxyphenyl arsenoxide; 3-nitrophenyl arsenoxide; 3,3'-diamino-6,6'-dimethoxyarsenobenzene-N,N'-dimethylene disodium sulfonate; and 3,3'-diamino-6,6'-dimethylarsenobenzene-N,N'-dimethylene disodium sulfonate. Data obtained so far indicate that 3-methoxyphenyl arsenoxide and 3-amino-4-hydroxyphenyl arsenoxide (mapharsen) have the same toxicity in normal as in Sarcoma 37 bearing female CAF₁ mice. The significance of these findings remains to be ascertained.

THE BACTERIAL CELL OF *S. MARCESCENS* AS A SOURCE OF TUMOR-NECROTIZING POLYSACCHARIDE. ADRIAN PERRAULT (by invitation), and M. J. SHEAR. (Chemotherapy Section, National Cancer Institute, Bethesda, Md.)

The bacterial cells, grown in a simple medium of inorganic salts and glucose, were removed from cultures of *S. marcescens* in a refrigerated Sharples supercentrifuge. A larger amount of tumor-necrotizing polysaccharide was obtained from the cells than from the filtrates of the same cultures. The bacterial paste sedimented in the centrifuge was diluted with 1 ml. of buffered saline for

each gram of material, and 0.2 M trichloroacetic acid was added in a volume sufficient to bring the final concentration of this acid to 0.1 M. Solid phenol was added to give a 5 per cent concentration. This mixture was shaken to a uniform milky suspension and placed in a refrigerator until sufficient quantities were accumulated for large scale fractionation.

The collected suspensions were centrifuged and the sediment discarded. One volume of hexane (boiling range 60 to 70° C.) and three volumes of absolute ethyl alcohol were added. After stirring 10 to 15 minutes a thick gel rose to the surface. The bottom layer was siphoned and discarded. The gel was treated with water, and NaOH was added with the pH kept below 10. From then on the fractionation paralleled the method used in isolating the tumor-necrotizing polysaccharide from the filtrates of the same cultures.

Bioassays in mice bearing Sarcoma 37 showed a tumor-necrotizing activity comparable to the polysaccharide obtained from the filtrates. A positive reaction for carbohydrates was obtained with Dreywood's anthrone reagent and with the Molish test. Benedict's solution was not reduced. Micro-Kjeldahl analysis gave a total nitrogen content of 1.6 per cent.

A STUDY OF THE HISTOGENESIS OF SARCOMA 37 IMPLANTS. C. H. U. CHU (by invitation) and R. C. MACCARDLE. (Chemotherapy Section, National Cancer Institute, Bethesda, Md.)

Intramuscular transplants of Sarcoma 37 from the right hind leg were introduced subcutaneously into normal mice. Muscle and connective tissue from the left hind leg of tumor-bearing and non-tumor-bearing litter mates were transplanted also subcutaneously to serve as controls. Serial sections were made to study the fate of such implants and the tissue reactions of the hosts from periods of 2 hours to 12 days after transplantation. Results seem to indicate that prior to vascularization (about the fourth day), tumor cells became necrotic. At the periphery of the necrotic implant, giant cells are invariably observed, the origin of which is not yet determined.

EXFOLIATIVE CYTOLOGY OF THE ORAL CAVITY. PAUL W. MONTGOMERY (Introduced by EMMERICH VON HAAN). (Department of Pathology, Ohio State University, Columbus, Ohio)

The exfoliative cytology of the oral cavity has been studied in order to investigate its usefulness for diagnostic purposes. By examining a large number of healthy individuals the normal patterns for the various regions of the mouth and for the various age and sex groups have been established. Statistical analysis of the figures obtained by counting the various cell types has proven the statistical significance of some of the differences observed. Various pathological conditions of the oral cavity, including malignant tumors, were studied. It could be shown that the methods of exfoliative cytology possess a limited value for the recognition of oral pathology.

DISCUSSION OF THE ELECTRONIC THEORY OF CARCINOGENESIS. A. R. T. DENUES (Introduced by WILLIAM L. SIMPSON). (Detroit Institute of Cancer Research, Detroit, Mich.)

The unique theoretical position of the thesis of Alberte Pullman and others, proposing correlation of electronic structure of chemical carcinogens with their biological potency, appears to justify more attention than is apparent in the literature. The results of studies, made with the help of specialists, of the validity of the general treatment are, therefore, presented together with some analyses of the trends in recent publications. Collectively, these seem to indicate that one is not compelled, on either theoretical or experimental grounds, to attach great cogency to this theory at present. However, in view of the fundamental nature of the interest and of its possible promise of a theoretical unification of carcinogenic mechanisms, work to determine the general validity of the proposal is to be encouraged. The orderly development of the theory, with biological testing of its predictions, as currently pursued in Paris, offers some hope of clarifying its importance. Other evidence may come from indirect experiments, such as determinations of relative carcinogenicities of hydrocarbon isologs with critical substitutions of deuterium for protium, to affect local kinetics without affecting the dominant parameter involving electron distribution.

COMPLEMENT FIXATION IN ANIMAL NEOPLASIA. I. A STUDY OF TECHNICS FOR MEASUREMENT OF THE REACTION IN RABBIT SERUM WITH SPECIAL REFERENCE TO THE TEMPERATURE OF INACTIVATION. HELEN THORNTON, LESTER D. ELLERBROOK, and MARK RHEES (by invitation), and STUART W. LIPPINCOTT. (Department of Pathology, School of Medicine, University of Washington and the Cancer Control Division, National Cancer Institute, Bethesda, Md.)

The extent of complement fixation upon the admixture of antigen and inactivated serum of New Zealand white rabbits bearing the Brown-Pearce carcinoma has been measured by the addition of graduated known amounts of complement to identical portions of the other reagents. Volumetric measurements of the amount of complement required were made at the endpoint of 50 per cent hemolysis. As a standard of reference for future antigen preparations the antigen employed was a centrifuged saline extract of the neoplasm.

When the sera were inactivated for 30 minutes at temperatures decreasing step-wise from 70° C. to 53° C. the amount of complement required for 50 per cent hemolysis in tests of 1 : 5 or 1 : 10 dilutions of fresh or previously frozen positive sera increased markedly and then decreased. Similar results were obtained with these sera in the absence of antigen although to a less marked degree. Fresh and frozen normal sera on the other hand tended to require slowly increasing amounts of complement although some of the fresh sera again required smaller amounts of complement when inactivated at 53° C. The absolute temperature difference

tended to be less marked with increased dilution of the sera.

These variations with the temperature of inactivation must be taken into account in the evaluation of the results of the tests.

THE EFFECT OF FOLIC ACID AND ANTI-FOLIC COMPOUNDS ON THE GROWTH OF CARCINOMA, SARCOMA, OSTEOGENIC SARCOMA, LYMPHOSARCOMA, AND MELANOMA IN ANIMALS. KANEMATSU SUGIURA and C. CHESTER STOCK. (Division of Experimental Chemotherapy, The Sloan-Kettering Institute for Cancer Research, New York, N.Y.)

An extensive study has been made on the effect of pteroyl glutamic acid (folie acid), 4-amino-pteroyl glutamic acid (Aminopterin), 4-amino-N₁₀-methyl pteroyl glutamic acid (A-Methopterin), 4-amino-pteroyl aspartic acid (Amino-An-Fol), and 2,6-diamino purine on the growth of Sarcoma 180, mammary adenocarcinoma EO 771, Harding-Passey melanoma, Wagner osteogenic sarcoma and Patterson lymphosarcoma in mice and Sarcoma R 39 and Flexner-Jobling carcinoma in rats. Subcutaneous inoculations of tumors into young animals were carried out by the usual trocar method. In general, the first intraperitoneal injection of compounds was given 1 to 7 days after tumor transplantation and injection was continued for 7 to 14 days.

The daily doses of 50 mg. per kg. of folic acid had no effect upon the growth of Sarcoma 180, adenocarcinoma EO 771, Harding-Passey melanoma, Wagner osteogenic sarcoma, Patterson lymphosarcoma, Sarcoma R 39 and Flexner-Jobling carcinoma in animals. Dipterin (100 mg./kg.) and Teropterin (400 mg./kg.) had no inhibitory effect on Sarcoma 180 and Sarcoma R 39. The daily doses of 0.25 mg. per kg. of Aminopterin had destructive effect on rat sarcoma, marked inhibitory effect on Sarcoma 180 and mouse lymphosarcoma, slight inhibitory effect on mouse adenocarcinoma and melanoma, but no effect on mouse osteogenic sarcoma and rat carcinoma. The daily doses of 1.5 to 2.0 mg. per kg. of A-Methopterin had destructive effect on rat sarcoma, marked inhibition on Sarcoma 180 and lymphosarcoma, slight inhibition on mouse adenocarcinoma and melanoma, but no effect on mouse osteogenic sarcoma and rat carcinoma. The daily doses of 45 to 50 mg. per kg. of Amino-An-Fol had destructive effect on mouse lymphosarcoma and rat sarcoma, marked inhibition on Sarcoma 180, slight inhibition on mouse adenocarcinoma, but no effect on mouse melanoma, mouse osteogenic sarcoma and rat carcinoma. The daily doses of 60 to 70 mg. per kg. of 2,6-diamino purine had marked inhibitory effect on rat sarcoma, but had no effect on Sarcoma 180, mouse adenocarcinoma and osteogenic sarcoma.

At effective levels, anti-follic compounds mentioned were toxic to the hosts, causing weight loss and many deaths. These animals showed intense diarrhea, marked reduction in the size of the spleen and number of erythrocytes in bone marrow.

Aminopterin and A-Methopterin produced neither

an inhibitory nor curative effect upon spontaneous breast cancers in mice.

FLUORESCENCE STUDIES OF CARCINOGENS IN RAT'S SKIN. PERIAN CAMBEL (by invitation). (Department of Anatomy, Division of Cancer Research, Washington University Medical School, St. Louis, Mo.)

The carcinogens, 20-methylcholanthrene, and 9,10-dimethyl-1,2 benzanthracene were applied in 0.6 benzene solutions to the interseapular shaved skins of adult rats and their subsequent distribution was observed by fluorescence microscopy. Both carcinogens enter the sebaceous glands as Simpson and Craner have reported in mice. However, even after 18 paintings the sebaceous glands of these rats persist, which is in sharp contrast to their early destruction in mice. This, and other differences between the fate of the carcinogens in these two species, will be discussed as possible factors in their differing response by cancer production.

THE EFFECT OF TUMORS ON ANTIBODY LEVELS IN MICE. D. R. A. WHARTON (Introduced by H. J. CREECH). (Lankenau Hospital Research Institute and The Institute for Cancer Research, Philadelphia 30, Pa.)

The effect of tumors on antibody levels resulting from injection of *Serratia marcescens* polysaccharide and other antigens has been studied in mice bearing spontaneous and transplanted tumors. Lower agglutinin levels were found in sera of Sarcoma 37 and Sarcoma 180-transplanted Swiss mice, than in sera of similarly treated normals. This might be due to: the tumor or the toxic polysaccharide acting upon the antibody-forming mechanism, or the destructive or inhibitory action of some substance elaborated by the tumor, or induced by it, which acts upon the antibody. Tumor-bearing mice injected with anti-polysaccharide mouse serum 4 days previously showed lower agglutinin levels than similarly treated controls. Apart from whatever action the tumor or polysaccharide might have upon the antibody-forming mechanism, the presence of the tumor appears responsible for development in mice of a substance destructive or inhibitory to antibody.

This substance was not demonstrable in: C3H or Swiss mice bearing spontaneous mammary tumors; a transplantable mammary carcinoma up to fifth transplant generation; a transplantable methylcholanthrene-induced fibrosarcoma. However, Barrett's C3HBA showed definite effect in the forty-ninth to fifty-third transplant generations.

Antipolysaccharide rabbit serum passed through Sarcoma 37 and normal mice showed no difference in titre. The destructive or inhibitory action produced in mice bearing certain tumors seems specific for mouse antibodies and not for rabbit antibodies.

Mice in which tumors have regressed yield antibody titres comparable with those of normal mice. Antibody-destroying, or inhibiting properties seem to disappear when tumors regress.

THE FLUORESCENT FRACTION OF HORSE SMEGMA AND THE POSSIBLE ROLE OF TRITERPENOIDS OF SEBUM-PRODUCING ORGANS IN CARCINOGENESIS. H. SOBEL and A. PLAUT (by invitation). (Laboratories of Beth Israel Hospital, New York, N.Y.)

The non-saponifiable fraction of horse smegma which had previously been shown to be carcinogenic (Plant and Kohn-Speyer) was separated into a fluorescent hydrocarbon-containing fraction (S1) and the remainder (S2). In S1 a substance giving a 2 banded fluorescent spectrum above 400 m μ was detected in minute amounts in 6 of 8 attempts. It cannot be stated whether this was naturally produced or an external contamination acquired during lifelong accumulation. A second fluorescent substance which was found in large amounts and which gave a broad-banded fluorescent spectrum below 400 m μ was shown to be a normal cyclization product of squalene which is present in abundant quantities. After nine months application to skin pockets of mice, S1 produced no tumors and S2 one papilloma.

A colorimetric method has been developed for the determination of squalene and this substance has been shown to be present in several sebum-like substances of human origin. It is suggested that aberrant cyclization of squalene in skin followed by aromatization could lead to the formation of a carcinogen. It was determined that rat sebum differs from human sebum and lanolin. Therefore, the composition of sebum may condition species response to painting with carcinogen. It is suggested that triterpenoids may play some role in carcinogenesis and anticarcinogenesis.

MECHANISMS OF METASTASIS. I. ZEIDMAN (Introduced by DALE REX COMAN). (Department of Pathology, University of Pennsylvania, School of Medicine, Philadelphia 4, Pa.)

Previous investigations in this laboratory on invasiveness of cancer were continued by a study of the conditions that determine the number of metastases from a transplanted tumor. The factors studied were size and number of transplanted tumors, and their duration. The transplantable fibrosarcoma, T-241, was inoculated into one or both flanks of C 57 black mice. Pairs of mice bearing single tumors and pairs bearing double tumors were sacrificed on the same day at intervals from 9 to 26 days after tumor inoculation. The tumor volumes were measured by fluid displacement, and the lung metastases were counted. There was no significant relationship between number of lung metastases and primary tumor volume. The number of lung metastases produced by two tumors in the same host was not different from the number of metastases produced by a single tumor. The correlation between number of metastases and duration of the primary tumor was significantly positive. In cancer of man, where hosts and tumors are variables, there is no apparent correlation between the size of the primary tumor and the number of metastases. Surprisingly, in the above experiments, where host and tumor variables were largely controlled, the same lack of correlation between size of primary tumor and number of metastases was apparent.

RESPONSE OF THE CENTRAL NERVOUS SYSTEM OF THE CHICKEN TO METHYLCHOLANTHRENE: FAILURE TO INDUCE TUMORS AFTER FOUR YEARS OF STIMULATION. WILLIAM O. RUSSELL and GEORGE S. LOQUVAM (by invitation). (The M. D. Anderson Hospital for Cancer Research, Houston, Texas)

Previous experiments indicating that tumors of nervous tissue origin could be regularly induced in the brains of rats and mice with methylcholanthrene and that in the rat a diet deficient in certain B vitamins influenced the induction period, suggested further study of the problem in fowls.

Pedigreed white leghorn chickens were selected for the experiment and pellets of 30 per cent methylcholanthrene fused with chemically pure cholesterol were implanted in the right cerebral hemisphere. A thiamine deficient diet was given at intervals to one group, followed by the injection of thiamine hydrochloride intramuscularly and a period of normal diet. It was only possible to give three periods of thiamine deficiency because the fowls were moved to another laboratory where it was not possible to continue the dietary phase of the problem. The periods of deficiency were given in a four month period. None of the chickens developed tumors. Six chickens were sacrificed 4 years and 8 months after implantation of the carcinogen; 10 chickens survived 3 years and 15 chickens survived 2 years. These results indicate that the central nervous system of the chicken is resistant to the carcinogenic stimulation of methylcholanthrene in concentrations that regularly produce neoplasms of the brain in rats and mice.

AN EXPERIMENTAL STUDY OF THE EFFECTS OF MALIGNANCY AND DIABETES ON EACH OTHER. ALAN W. CARRIE (by invitation) and ARTHUR W. HAM. (Department of Anatomy, University of Toronto, Toronto, Canada.)

Sarcoma 37 was transplanted into rats made diabetic with alloxan and into normal rats. Tumors were observed to grow just as rapidly in the diabetic rats as in the non-diabetic ones. The blood sugar level was followed for 2 months in 12 diabetic rats with growing tumors and in 13 normal rats with comparable diabetes. No significant difference between blood sugar levels in the two groups was observed. A growing tumor in a diabetic animal was not found to affect the rate at which its blood sugar level fell on starvation.

The fact that tumors were found to grow just as quickly in diabetic rats as in non-diabetic controls suggests that the metabolic processes of the malignant cell are not dependent on insulin. Since tumor cells obtain a large proportion of their energy requirements from fermentation, these findings suggest further that none of the reactions concerned in glycolysis are dependent on insulin. Nevertheless, even though tumors can glycolyze sugar in the absence of insulin this experiment indicates that the demands of a tumor on an organism for sugar are not sufficient to substantially lower its blood sugar level and hence that the diminution of glycosuria noted in diabetics (before the advent of insulin) when they

developed malignant tumors is probably not to be explained by the tumors, as a result of their glycolytic activities, having substantially reduced the blood sugar levels of the individuals concerned.

THE INFLUENCE OF CERTAIN SULFONAMIDE DRUGS ON CANCER SUSCEPTIBILITY AND REPRODUCTION IN MICE.* FRANK H. J. FIGGE,[†] and GERALDINE F. WOLFE, ROSALIE YERKES FIGGE (by invitation). (University of Maryland School of Medicine, Baltimore 1, Md.)

The influence of sulfanilamide, sulfathiazole, and sulfaipyridine on the cancer susceptibility of mice was tested over a five year period. A total of 4853 mice of various strains (C3H, C57, A) were treated throughout life with the various sulfonamide drugs. In some cases, the experimental period involved 8 generations of mice receiving continuous treatment. No significant effect of sulfonamide treatment on cancer susceptibility was observed, but it was found that sulfanilamide in a concentration of 3 grams per liter in the drinking water inhibited reproduction. The inhibitory influence of sulfaipyridine on reproduction was not so marked and did not extinguish the lines until the third- or fourth-treated generation. Sulfathiazole had little or no effect on reproduction and the mice treated continuously with this drug were followed through 8 generations.

An attempt was made to investigate the nature of this inhibition of reproduction by sulfanilamide. The reproduction inhibiting potentialities of sulfonamide drugs appeared to parallel the tendency of the various drugs to produce Heinz bodies in the erythrocytes of mice; but this parallelism may be coincidental. The inhibition of reproduction by sulfanilamide could not be counteracted by administration of large supplements of various vitamins to the diet. Feeding thyroid also failed to produce any appreciable change in the reproductive ability of sulfanilamide-treated mice.

FURTHER STUDIES ON HYPERVOLEMIA AND ASSOCIATED CHANGES IN MICE BEARING A TRANSPLANTED GRANULOSA-CELL TUMOR. JAMES T. WOLSTENHOLME (Introduced by W. U. GARDNER). (Department of Anatomy, Yale University School of Medicine, New Haven, Conn.)

Mice bearing a transplanted granulosa cell tumor have hypervolemia and dilatation of the sinusoids of the liver, spleen and adrenal glands. (Furth, Boon, and Sobel, 1945.)

Four groups of experiments were set up:

Parabiotic series.—Animals of the C57 strain were castrated at 60 to 90 days of age and small fragments of a granulosa cell tumor were transplanted subcutaneously. When the tumor transplant was approximately 3 to 4 mm. in diameter, the animal was parabiosed to a littermate. When the tumor attained 1 mm. in diameter or

* Aided by grants from the Anna Fuller Fund and the Donner Foundation.

† Visiting Professor of Anatomy, University of Pennsylvania, School of Medicine, Philadelphia, Pennsylvania.

more, the tumor bearing animal had hypervolemia and liver changes; no changes occurred in the non-tumor parabiont twin.

Splenectomy series.—Small fragments of a granulosa cell tumor were transplanted subcutaneously into castrated animals of the C57 strain. When the tumor transplant was 3 to 8 mm. in diameter the animal was splenectomized. These animals developed hypervolemia and liver changes.

Tumor removal experiment.—Eight mature C57 animals bearing granulosa cell tumors of different sizes and having hypervolemia and liver changes had the tumors removed. When sacrificed 5 days later there was no hypervolemia or liver change present.

Tumor extract experiment.—Eighty-three grains of fresh granulosa cell tissue obtained from C57 mice were extracted with acetone. The acetone extract was evaporated and redissolved in sesame oil. The acetone insoluble fraction was dried, powdered and suspended in water. Daily injections of the acetone extract into C57 animals and daily injections of the aqueous suspension into C57 animals for an 8 week period failed to produce the hypervolemia or associated changes that were produced by the tumor.

EFFECTS OF A NITROGEN MUSTARD ON THE CICHLID FISH, *TILAPIA MACROCEPHALA* (BLEEKER). SOPHIE JAKOWSKA (by invitation), and R. F. NIGRELLI. (College of Mount St. Vincent and New York Zoological Society, New York, N.Y.)

Young mature fish, 4 to 6 cm. in standard length, from a common stock were placed in tanks containing 4 liters of homotypic conditioned water in which was dissolved enough methyl-bis (beta-chloroethyl) amine hydrochloride to make concentrations of 0.0017 and 0.002 per cent. The tanks, each containing 4 fish, were continuously aerated and kept at room temperature. The fish showed hyperemic gills at the end of 24 hours. At the end of 48 hours, desquamation of the epithelium of the gills, oral and branchial cavities was evident and there also occurred a general nephrosis with glomerular and tubular degeneration. In all fish there was an invasion by macrophages and eosinophiles in the tissues adjacent to the branchial region in response to a parasitic infection by the ciliate, *Ichthyophthirius multifilis*. In treated fish, the parasites were absent but the massed macrophages and eosinophiles were still present, indicating that the nitrogen mustard, in the concentration and time used, had no apparent effect on these cells. With the exception of the testis, the other internal organs including the blood, showed no striking histological changes. Feulgen-stained smears of the testis showed that divisions were arrested, since only cells in the resting stage and in prophase were observed, whereas in untreated fish all stages of mitosis and meiosis were encountered. Fish exposed to 0.01 per cent concentration for 1 hour did not show any significant tissue changes, indicating that the effects of the nitrogen mustard, as employed under these conditions, were manifest only after comparatively longer exposures.

THE QUESTION OF A THYROID GROWTH CENTER. JOHN H. VAN DYKE. (Indiana University School of Medicine, Bloomington and Indianapolis, Ind.)

During development in mammals an ultimobranchial body, derived from the hind end of the embryonic pharynx, comes to lie within the center of each lateral lobe of the thyroid where apparently it is frequently induced to form functional thyroid parenchyma.

In rats after birth ultimobranchial tissue, normally indistinguishable from thyroid tissue, may be similarly modified by feeding vitamin A deficient diet, administration of estrogen or methylcholanthrene. Following early hyperplasia, this tissue usually transforms, through metaplasia, into multiple cysts lined by stratified squamous epithelium whereas adjacent peripheral thyroid follicles undergo general atrophy. Normal old age rats frequently exhibit this condition; not infrequently associated with cystadenomata.

In rats, the site of maximal mitoses appears to be in the center of each thyroid lobe, both in normal growing animals and during experimental hyperplasias. When thyroids containing ultimobranchial cysts are stimulated spontaneously (sheep) or, under optimum conditions, in rats by cold or hypophyseal extract, mitotic division of thyroid parenchyma does not ensue. Apparently, however, there is a compensatory proliferation of masses of relatively indifferent cells from the bases of ultimobranchial cysts which transform, upon demand, into typical thyroid tissue in a manner to be described.

Evidence suggests that ultimobranchial tissue is plastic, normally indistinguishable from thyroid tissue, and may function after birth as a thyroid growth center or, being labile, may undergo cyclic phenomena depending upon factors altering thyroid activity. Conditions augmenting thyroid hyperactivity in mammals (including man), particularly those with ultimobranchial lesions, may predispose to certain compensatory neoplasms—especially during senility.

RESULTS OF THE BIOLOGICAL TEST FOR MALIGNANCY. HOWARD H. BEARD. (Cancer Clinic, Holy Cross Hospital, Chicago, Ill.)

Four hundred urines from malignant patients (biopsy in 36 per cent) were run by the biological test and 94 per cent of them gave positive results. These were from 55 different sites (breast, 50; uterus, 39; stomach, 33; lungs, 27; cervix, 26; colon, 16; leukemia, 20; etc.). In 221 nonmalignant urines there were 87 per cent negative tests. In 183 miscellaneous urines there were 62 or 34 per cent positive and of these 11 were from pregnancy and postpartum urines. In 67 normal urines 97 per cent were negative. These results agree closely with those of Roffo (1), Sakai (2), and Aron (3), and further

confirm the validity of the trophoblastic thesis of malignancy. The following applications of the biological test were suggested: (a) in supplementing clinical and pathological diagnosis of malignancy, (b) in testing for the presence or absence of metastases after treatment, (c) in controlling chymotrypsin therapy, (d) in distinguishing between the presence of a benign and malignant tumor in the patient, and (e) in detecting the presence of early malignancy in a given individual. A positive test in a malignant patient will become negative after 2 or 3 weeks of chymotrypsin therapy (total from 100 to 300 mgs.) in those patients who survive. In those that don't the test may remain positive in spite of enzyme therapy. Another investigator has discovered the presence of a chymotrypsin inhibitor only in the sera of pregnant women and malignant patients. The antichymotrypsin and biological tests agreed closely in 43 out of 46 cases.

1. Roffo, A. H. Bol. Inst. Med. Exper., No. 65, 419, 1944.
2. Sakai, S. Igaku Kenkyu, 13, 1, 1939.
3. Aron, M., Presse med., 43, 1044, 1935.

CARCINOGENIC ACTION OF CERTAIN CATALYTICALLY CRACKED OILS WITH HIGH BOILING POINTS. KANEMATSU SUGIURA, WILLIAM E. SMITH, AND DOUGLAS SUNDERLAND (by invitation). (Sloan-Kettering Institute for Cancer Research, New York, N.Y.)

In collaboration with a petroleum company* 200 samples of oils, waxes, and tars were tested for carcinogenicity by painting upon the skin of animals. Samples of petroleum submitted to catalytic cracking at high temperatures exhibited marked carcinogenicity.

Mice painted with high boiling catalytically cracked oils showed, in certain cases, papillomas as early as 29 days and squamous cell carcinomas as early as 70 days. With continued painting, papillomas developed in nearly every animal and in a majority underwent malignant change. The same material elicited papillomas in all of 6 rhesus monkeys after 3 years of painting, but no tumors were obtained in guinea pigs and only 1 in 59 rats living over 1 year. Papillomas arose in all and cancers in 3 of 26 rabbits.

Certain samples of these catalytically cracked oils showed a carcinogenic potency nearly comparable to 0.3 per cent solutions of methylcholanthrene in acetone. The carcinogenic material was in the fractions distilling over above 700° F. The carcinogenic material can be concentrated in the aromatic fractions after discarding heptane-insoluble matter and adsorbing on silica gel columns followed by elution with cumene or acetone.

* These studies were carried out under a grant from the Standard Oil Company (New Jersey) and certain affiliates in cooperation with the Medical Department of that Company. All samples were prepared and supplied by the Standard Oil Development Company.

American Association for Cancer Research, Inc.

40th Annual Meeting

Hotel Fort Shelby, Detroit, Michigan

April 16 and 17, 1949

Proceedings of Business Sessions

MINUTES OF THE MEETING OF THE BOARD OF DIRECTORS HELD APRIL 15, 1949

The meeting was held in the Commerce Room of the Hotel Fort Shelby in Detroit, Michigan, and was called to order at 7:05 p.m. by the Chairman of the Board. Present were Board members J. C. Aub, J. J. Bittner, A. M. Brues, E. V. Cowdry, E. A. Doisy, J. Furth, C. W. Hooker, Charles Huggins, and H. C. Taylor, Jr., and Dr. Theodore P. Eberhard, Business Manager of *CANCER RESEARCH*.

The minutes of the last meeting were read and approved.

REPORTS OF OFFICERS

The President reported that the chief interest of his office had been in *CANCER RESEARCH*, pursuant to the agreement at the last meeting that the Association assume responsibility for the journal. At that time *CANCER RESEARCH* was several months in arrears of publication and its editor wished to be relieved of his duties. Acting with the approval of Mr. W. H. Donner of Donner Foundation, Cancer Research, Inc., a holding company consisting by definition of the three officers of the Association, was incorporated in Wilmington, Delaware on June 17, 1948. On September 19, 1948 a meeting of the Board of Managers of *CANCER RESEARCH*, appointed by the Board of Directors of the Association, was held in Washington. Present were Drs. Balduin Lucké, P. E. Steiner, E. W. Shrigley, Albert Tannenbaum, Theodore P. Eberhard, and Charles Huggins. After considering estimates of several publishing houses, the Board of Managers agreed upon the University of Chicago Press as Printer and Dr. Paul E. Steiner as Editor-in-Chief. Both Doctor Steiner and the Press accepted their appointments. The Board of Managers recommended that the "abstracts section" in *CANCER RESEARCH* be discontinued. Applications for grants in aid for 1949 were then made to the Jane Coffin Childs Fund for \$5,500; the Anna Fuller Fund for \$2,000; and the Pardee Foundation for \$1,000. These organizations generously agreed to these subsidies, thus meeting the conditions set by Mr. W. H. Donner for his subsidy of \$5,000 for 1949. The annual cost of publication is roughly \$25,000. On October 25, 1948 an agreement was reached between the University of Chicago Press and Cancer Research, Inc. for publication until

January 1, 1951, and on a year to year basis thereafter, with the understanding that after January 1, 1951 either party may terminate the arrangement on ninety days notice. Due to the effective work of Dr. Lucké and his staff, ably aided by Doctor Eberhard, the Ann Arbor Press has completed publication of Volume 8, with the exception of the December issue which is now ready for final printing. On November 22, 1948 the Ann Arbor Press agreed to terminate its contract with Cancer Research, Inc. on completion of Volume 8. On January 25, 1949 the January 1949 number of *CANCER RESEARCH* appeared under the aegis of Dr. P. E. Steiner's new editorial board and the new printer. Since then issues have appeared regularly and on schedule. The Press deals with subscriptions to back volumes and to current subscriptions of non-members. The Press is also investigating the possibility of obtaining advertising, but they consider advertising inadvisable until subscriptions total at least 1500. The present circulation is approximately 1300. When the circulation reaches 2500, advertising can provide a good source of support. On March 25, 1949 Editor-in-Chief Steiner notified Cancer Research, Inc. that he will not be available as Editor upon completion of Volume 9. On April 1, 1949 the monies of Cancer Research, Inc. on deposit with the Fidelity-Philadelphia Trust Company totalled \$19,620.05. We are now publishing a journal of 64 pages that is appearing promptly. Cancer Research, Inc. and the Board of Managers of *CANCER RESEARCH* believe that the cost of the journal to subscribers and to members of the Association is too low, and they recommend an increase to both classes of subscribers. The President reiterated his conviction that the publication of *CANCER RESEARCH* is a vital function of the Association. He emphasized that Cancer Research, Inc. is indebted particularly to Balduin Lucké, Paul E. Steiner, and Theodore P. Eberhard for their heroic efforts to transfuse and transform *CANCER RESEARCH*; that we are singularly in the debt of Mr. William H. Donner, the Childs and Fuller Funds, and the Pardee Foundation, without whose assistance nothing could have been attempted; that Cancer Research, Inc. is most grateful to the other members of the Association for their patience and support in this trying period.

The President concluded his report by restating his

conviction that the Association is a non-political group, organized to disseminate information, and to provide annual opportunity for meeting for comfort, encouragement, and exchange of ideas in a convivial atmosphere.

It was voted that the report of the President be accepted with commendation.

The President, in the absence of Dr. Baldwin Lucké, then presented the report of the Board of Managers of *CANCER RESEARCH*. The report recommended the abolition of the Board of Managers and its replacement by an Executive Council to be composed of the immediate Past President, President, Vice-President, and Secretary-Treasurer of the Association, and the Editor-in-Chief of *CANCER RESEARCH*. It was recommended that the Executive Council handle administrative problems of the journal and problems of the Association needing prompt action in the interval between annual meetings of the Association and its Board of Directors; that editorial policies of the journal be governed by the Editorial Board of the Journal. After brief discussion, it was voted that the recommendations of the Board of

Managers of *CANCER RESEARCH* be adopted. The report of the Board of Managers also urged increasing annual dues of active members to ten dollars, eight dollars of which would go to *CANCER RESEARCH* and two dollars of which would be applied to the expenses of the Association. It was voted to accept the recommendation and to refer it to the members of the Association at their meeting.

It was then voted that the Secretary appropriately thank the Editors of *CANCER RESEARCH* on behalf of the Association for their effective labors in bringing out *CANCER RESEARCH*.

The Secretary reported that Dr. Vasant Ramji Khanolkar of Bombay, India had accepted the Honorary Membership to which he was elected at the last annual meeting.

The Secretary reported that comparatively little objection to the increase in annual dues from three to seven dollars had come to his attention. One member proposed to the Secretary that two classes of membership be created, full membership corresponding to the pres-

TREASURER'S REPORT

March 31, 1949

Journal Fund

On deposit (Union and New Haven Trust Co.) 3-1-48
Interest, 3/1/48 to 3/31/49

\$ 81.00
80
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\$ 81.80

Savings Account

On deposit (Greenwich Savings Bank) 3-1-48
Interest, 3/1/48 to 3/31/49

654.34
9.84
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664.18

Operating Funds

On deposit (Union and New Haven Trust Co.) 3-1-48
Receipts, Dues
Subscriptions to back volumes *Cancer Research*

2,775.40
3,856.20
45.00
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664.18

Disbursements

Cancer Research, contribution, 1948
Cancer Research, subscription 1948, one member
University of Chicago Press
464 subscriptions @ \$6.00
4 supplements to paid subscriptions
2 subscriptions @ \$6.00, less \$5.00 credit
Overpayment
Traveling expenses, Board of Managers, *Cancer Research*
Annual Meeting, 1948
Printing (Programs, etc.)
Slide projectors, services
Telephone, shipping charges
Secretary's office
Secretarial assistance
Printing, office supplies
Postage, stamps
Addressograph plates (605), 3 drawers
Refunds on overpaid dues
Bank charge, Canadian check
Reprints, minutes of Directors' meeting Oct. 13, 1946

\$ 602.00
5.00

\$2,784.00

4.00

7.00

12.00 2,807.00

136.21

2,775.40
3,856.20
45.00
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6,676.60

160.75

140.30

6.17 313.92

250.00

180.14

63.91

51.11 545.16

5.00

.27

.55

BALANCE, March 31, 1949

4,455.09

\$2,221.51

I hereby certify that the accounts and vouchers in the American Association for Cancer Research, Inc., for the above recorded period, have been examined by me, and that the above are true statements of its financial operations and of its financial conditions as of March 31, 1949.

TREODORE P. EBERHARD
Auditor for the Directors

ent active membership and associate membership not carrying with it a subscription to *CANCER RESEARCH* and costing three dollars annually. Another member raised the question whether a husband and wife, both members of the Association, should both pay full dues and both receive *CANCER RESEARCH*. To date 470 members have paid dues for 1949. The request of several members that a divided statement of dues be rendered, one that will allow these members to pay what they consider to be their dues and their laboratory to pay for the journal, was mentioned. The Secretary raised the question whether he has the authority to meet these requests. After brief discussion the following actions were taken. With respect to the establishment of an associate membership, it was voted that the By-Laws be affirmed. It was agreed with respect to full payment of dues by husband and wife that no special arrangements should be made at present. With reference to preparing divided statements, the Board voted "that the Secretary be instructed to issue a lump-sum statement of dues," the sense of the group being that dues are a set sum in return for which the member receives various rights and privileges, including *CANCER RESEARCH*, and that reimbursement is the problem of the member.

The Treasurer's annual report was then read and accepted, pending approval by an auditor. Dr. Theodore P. Eberhard was appointed auditor.

REPORTS OF STANDING COMMITTEES

Program Committee.—Chairman J. C. Aub reported that the number of abstracts submitted greatly exceeded that of any year in the past, and that his Committee attempted to place as many different investigators on the program as possible. Even with a two-day meeting with three sessions running concurrently, one-third of the papers submitted had to be read by title. In the discussion it was agreed that papers read by title are to be a permanent portion of the program. With respect to the next meeting, the Board voted to hold a three-day meeting to be held more or less in conjunction with the meeting of the Federation of American Societies for Experimental Biology, the precise time to be determined by the Program Committee.

Nominating Committee.—Chairman Hooker reported that his Committee had proposed for new members of the Board of Directors Drs. J. C. Aub, E. V. Cowdry, E. A. Doisy, W. U. Gardner, H. P. Rusch, M. B. Shlkin, H. C. Taylor, Jr., and Albert Tannenbaum. These names were listed on the proxy ballots sent to the members of the Association by the Secretary. Count of the ballots revealed that Doctors Aub, Cowdry, Doisy, and Gardner received the largest number of votes. The Board voted, "That the Secretary cast one ballot for the nominees chosen by the members." The new Directors were then declared elected.

Membership Committee.—Chairman Jacob Furth reported that the Association now has 604 active members, 4 emeritus members, 3 contributing members, and 8 honorary members. The resignations of the following members were submitted and accepted:

C. J. Christy	Catharine Macfarlane
Arthur F. Coca	William C. McCarty
Charles A. Elsberg	Angelo H. Roffo
William R. Franks	Isabel M. Scharnagel
J. E. Gendreau	Fritz Schlenk
A. W. Hengerer	Harold A. Solomon
Martha Elizabeth Howe	William H. Wehr
Richard Lewisohn	Florence R. White
	Thomas N. White

The requests of Dr. C. H. Bunting, Dr. B. J. Clawson, Dr. Thomas S. Cullen, and Dr. E. E. Tyzzer for transfer to Emeritus Membership were submitted and approved.

The deaths of the following members were announced with expressions of profound regret.

Montrose T. Burrows
Mortimer Cohen
Joseph McFarland
A. A. Thibaudeau

The nominations for active membership were then presented. The Committee recommended the election of 66 nominees. They were:

ARNESEN, AXEL NORMAN, M.D., Washington University School of Medicine, St. Louis 8, Mo.
BERENBLUM, ISAAC, M.D., National Cancer Institute, Bethesda 14, Md.
BODANSKY, OSCAR, Ph.D., M.D., Memorial Hospital, 444 East 68th Street, New York 21, N.Y.
DE BRUYN, WILLEMINA M., Ph.D., Johns Hopkins Hospital, Baltimore 5, Md.
BURCHENAL, JOSEPH H., M.D., Sloan-Kettering Institute for Cancer Research, 444 East 68th Street, New York 21, New York.
CAMBEL, PERIAN, M.D., Washington University School of Medicine, St. Louis 10, Mo.
CHALKLEY, HAROLD W., Ph.D., National Cancer Institute, Bethesda 14, Md.
CLARK, R. LEE, JR., M.D., M.D. Anderson Hospital, 2310 Baldwin Street, Houston 6, Texas.
COOK, ELTON S., Ph.D., Institutum Divi Thomae, 1842 Madison Road, Cincinnati 6, Ohio.
COPELAND, D. H., B.S., Alabama Polytechnic Institute, Auburn, Alabama.
DAVIDSON, ISRAEL, M.D., Mt. Sinai Hospital, 2750 West 15th Place, Chicago 8, Ill.
ELMAN, ROBERT, M.D., Washington University School of Medicine, St. Louis 10, Mo.
EVERETT, MARIE R., University of Oklahoma School of Medicine, Oklahoma City 4, Okla.
FISHMAN, WILLIAM H., Ph.D., Tufts College Medical School, Boston 11, Mass.
FRANTZ, IVAN DERAY, M.D., Massachusetts General Hospital, Boston 14, Mass.
GORBMAN, AUBREY, Ph.D., Barnard College, Columbia University, New York 27, N.Y.
GORHAM, L. WHITTINGTON, M.D., Albany Medical College, Albany 1, N.Y.
GREENBERG, DAVID M., Ph.D., University of California Medical School, Berkeley 4, Calif.

- GMER, ROBERT S., M.D., Massachusetts General Hospital, Boston 14, Mass.
- GRIFFIN, AMOS CLARK, Ph.D., Stanford University, Stanford, Calif.
- HAINLY, MORRIS H., Ph.D., New York University, New York 3, N.Y.
- HEIDELBERGER, CHARLES, Ph.D., University of Wisconsin, Madison 6, Wis.
- HOPPS, HOWARD C., M.D., University of Oklahoma School of Medicine, Oklahoma City, Okla.
- HUGULEY, CHARLES M., Jr., M.D., Emory University School of Medicine, Emory University, Ga.
- JONES, RALPH, Jr., M.D., University of Pennsylvania Hospital, Philadelphia 4, Pa.
- KENNEDY, BYRL J., M.D., Massachusetts General Hospital, Boston 14, Mass.
- KING, JOSEPH T., Ph.D., M.D., University of Minnesota, Minneapolis 14, Minn.
- KREKE, CORNELIUS W., Ph.D., Institutum Divi Thomae, 1842 Madison Road, Cincinnati 6, Ohio.
- LANSING, ALBERT L., Ph.D., Washington University School of Medicine, St. Louis 10, Mo.
- LAWRENCE, EDWIN A., M.D., University of Utah College of Medicine, Salt Lake City 5, Utah.
- LEATHEM, JAMES H., Ph.D., Rutgers University, New Brunswick, N.J.
- LENTA, SISTER M. PETRA, M.S., College of St. Scholastica, Duluth 2, Minn.
- LEWIS, GEORGE T., Ph.D., Emory University School of Medicine, Emory University, Ga.
- LUCK, J. MURRAY, Ph.D., Stanford University, Stanford, Calif.
- LUSHBAUGH, CLARENCE C., Ph.D., M.D., Los Alamos Scientific Laboratory, Los Alamos, New Mexico.
- MAUN, MARK E., M.D., St. Mary's Hospital, 1420 St. Antoine, Detroit 26, Mich.
- MELLORS, ROBERT C., Ph.D., M.D., Sloan-Kettering Institute for Cancer Research, 444 East 68th Street, New York 21, N.Y.
- MOORE, ALICE E., M.D., Sloan-Kettering Institute for Cancer Research, 444 East 68th Street, New York 21, N.Y.
- MULLIGAN, RICHARD M., M.D., University of Colorado School of Medicine, Denver 7, Col.
- NEWTON, BERNE L., M.D., Baylor University College of Medicine, Houston 1, Texas.
- NOVIKOFF, ALEX B., Ph.D., University of Vermont College of Medicine, Burlington, Vt.
- NUTINI, LEO G., M.D., Institutum Divi Thomae, 1842 Madison Road, Cincinnati 6, Ohio.
- ONELL, LESTER D., M.D., University of Chicago, Chicago 37, Ill.
- OPPENHEIM, ABRAHAM, M.D., 30 East 60th Street, New York 22, N.Y.
- PALETTA, FRANCIS X., M.D., 634 N. Grand, St. Louis 3, Mo.
- PETERMANN, MARY LOCKE, Ph.D., Sloan-Kettering Institute for Cancer Research, 444 East 68th Street, New York 21, N.Y.
- PINKERTON, HENRY, M.D., St. Louis University School of Medicine, St. Louis 4, Mo.
- RILEY, VERNON T., National Cancer Institute, Bethesda 14, Md.
- ROSENTHAL, THEODORE B., Ph.D., Washington University School of Medicine, St. Louis 10, Mo.
- RUGG, ROBERTS, Ph.D., Columbia University, New York 32, N.Y.
- SALMON, WILLIAM D., M.A., Alabama Polytechnic Institute, Auburn, Ala.
- SCOTT, WILLIAM W., Ph.D., M.D., Johns Hopkins Hospital, Baltimore 5, Md.
- SHACTER, BERNARD, Ph.D., Laguna Honda Home, San Francisco 16, Calif.
- SHAY, HARRY, M.D., Temple University School of Medicine, Philadelphia, Pa.
- SHETLAR, MARVIN R., Ph.D., University of Oklahoma School of Medicine, Oklahoma City 4, Okla.
- SKAPIER, JOSEPH, M.D., Ph.D., 330 West 72nd Street, New York 23, N.Y.
- STERN, KURT G., Ph.D., Polytechnic Institute of Brooklyn, Brooklyn 2, N.Y.
- STEVENS, CHARLES D., Ph.D., University of Cincinnati College of Medicine, Cincinnati 19, Ohio.
- SUMMIERSON, WILLIAM H., Ph.D., Army Chemical Center, Md.
- SYMEONIDIS, ALEXANDER, M.D., Ph.D., National Cancer Institute, Bethesda 14, Md.
- THURINGER, JOSEPH, M.D., University of Oklahoma School of Medicine, Oklahoma City 4, Okla.
- WARD, GRANT E., M.D., 214 Medical Arts Building, Baltimore 1, Md.
- WARTMEN, WILLIAM B., M.D., Northwestern University Medical School, Chicago 11, Ill.
- WIGHT, KENT M., Ph.D., National Cancer Institute, Bethesda 14, Md.
- WILSON, J. WALTER, Ph.D., Brown University, Providence 12, R.I.
- ZEIDMAN, IRVING, M.D., University of Pennsylvania School of Medicine, Philadelphia 4, Pa.

Committee on Cancer Research, Its Organization and Support.—The report, submitted by Chairman Shields Warren, has been filed in the office of the Secretary. By order of the Board an abstract of the report is given here:

The most striking development since the last report has been the establishment of a program for cancer research in the field of atomic energy. The initial appropriation made by Congress to the Atomic Energy Commission had \$4,000,000 earmarked for cancer research. The program has consisted of development of facilities for clinical investigation in conjunction with the national laboratories; provision of funds for the Atomic Casualty Committee of the National Research Council for study of the carcinogenic potentiality of a single dose of radiation in the human material at Nagasaki and Hiroshima; provision of funds for research in certain fields having to do with the utilization of radioactive isotopes for research in therapy; advancement of funds to the Office of Naval Research to permit continuation of cancer research projects threatened by the insufficient funds of the Office of

Naval Research; making available free in April, 1948 the isotopes P^{32} , I^{131} , and Na^{24} for cancer research and treatment; making available free in February 1949 all other isotopes for cancer research. Only shipping and handling charges must be paid by the investigator. For this purpose the Atomic Energy Commission has set aside \$450,000. The Isotope Distribution Committee of the Atomic Energy Commission and its Subcommittee on Human Allocations are doing effective work in making isotopes widely available and in insuring the safety of those who may come in contact with them.

A second important development is the initiation of a program of construction of research facilities by the U.S. Public Health Service. The program is the outgrowth of recognition by the Public Health Service that universities have insufficient funds for new construction, that present facilities are overcrowded, and that effective use of the brains and money available for cancer research is contingent upon space to work. The first step was authorized by the Eightieth Congress, and that policy is apparently to be continued by the present Congress.

A significant trend toward increased emphasis on chemotherapy is pointed out.

It is noted that most of the privately endowed foundations are now placing little emphasis on cancer, recognizing the sums provided by other sources. The foundations specifically for cancer, such as the Anna Fuller Fund and the Childs Fund, continue their effective support. The M. D. Anderson Foundation is gradually developing a potentially effective cancer center in Houston, and we expect this Foundation to be a source of future impetus to cancer research.

The report records the establishment of the new journal *CANCER*, and expresses satisfaction with the assumption of responsibility for *CANCER RESEARCH* by the Association. The publication of the "Index to Literature of Experimental Cancer Research, 1900 to 1933" by the Donner Foundation is regarded an important service to investigators.

The great number of meetings on various aspects of the cancer problem held since the war is mentioned. The Committee recognized the value of these meetings, but suggests that there is the possibility of too many meetings retarding rather than expediting research. The growing international co-operation in cancer research, however, is enthusiastically endorsed.

The reconstruction of the Roscoe B. Jackson Memorial Laboratory is recorded as being of great significance.

As to the future, the report considers it obvious that increased clinical research on patients is needed. The recent advancements in the laboratory need to be checked by pilot clinical studies. A great need is research wards whose cost will not be met directly by the hospitals. Significant exploration in this field is being made by the U.S. Public Health

Service. The fact that project research of a short-term character is not fully effective is again urged. The advantages of the institutional type of grant as developed by the American Cancer Society are mentioned. The occasional tendency within the recipient institution to break up the grant into a series of project grants is decried.

The report concludes with a restatement of the increased costs of research and of the fact that much current research is a painstaking survey of fields already roughly mapped out. Costly apparatus and teams composed of men familiar with a number of diverse scientific fields are needed.

REPORTS OF SPECIAL COMMITTEES

Committee on Local Sections of the Association.—Chairman E. V. Cowdry reviewed the survey made by his Committee and their recommendations, and presented from his Committee the following resolution to serve as a guide for an addition to the By-Laws of the Association:

"The American Association for Cancer Research may have local chapters in such areas which have sufficient numbers of individuals with scientific interest in cancer research. Application for establishment of the chapters shall be made by the local group of no less than 10 members of the Association, and shall be considered by the Board of Directors; if the Board agrees that the geographic area and other conditions are acceptable, the authorization for the chapter shall be made by the vote of the members at their annual meeting. Additional members of the chapter do not necessarily have to be members of the Association, if they meet the same general qualifications as members of the Association, with the exception of the publication requirement. Meetings of the chapters shall be held at least twice a year, and reports of such meetings shall be made to the Secretary of the Association."

The Board voted to accept the report and to adopt the resolution.

REPORTS OF REPRESENTATIVES OF THE CORPORATION

Dr. E. V. Cowdry, delegate to the International Cancer Research Congress, reported that a clarification of the Congress is expected at the meeting in Paris in the summer.

It was voted "That Drs. E. V. Cowdry and W. U. Gardner be recommended by the Association as official delegates of the United States to the International Cancer Research Commission, the recommendation to be transmitted by the Secretary to Dr. Ignacio Y. Milan in Mexico by order of the President of the Association."

UNFINISHED BUSINESS

After brief discussion it was voted not to affiliate with the Union of Biological Societies at the present time.

It was reported that a history of the Association is now in preparation.

NEW BUSINESS

In view of the inconstancy in the date of the annual meeting from year to year and because of the increased financial operations of the Association, it was voted, "That the fiscal year of the Association henceforth cover the period January 1 through December 31." The Board also voted to approve the briefest possible summary of activities in the published version of the Treasurer's report.

Authorization was given the Secretary-Treasurer to make such alterations in the composition of the annual statements of dues as are necessary.

Dr. E. V. Cowdry reported that plans have been made for the Fifth International Cancer Research Congress to be held in Paris in 1950, probably in July. Invitations have already been sent, and the President will be Dr. A. Lacassagne. It has been agreed to request the International Congress to send every member of the Association an invitation to present a paper.

It was pointed out that in the voting of members for new members of the Board of Directors during the last two years the largest vote has been cast for the first four names on the ballot. It was also pointed out that it has become customary for the Nominating Committee to renominate the retiring members of the Board, that any new Committee and any retiring Director is thrown into embarrassing circumstances if an unintentionally self-perpetuating Board is to be made impossible. After discussion it was voted, "That the order of names on the ballot for Directors be determined by lottery, and that this fact be stated on the ballot." It was further voted that "Beginning in 1951 no member of the Board of Directors can succeed himself in office for a period of one year."

Nominations for officers of the Association for the coming year were then made; for President, Joseph C. Aub; for Vice-President, Jacob Furth, for Secretary-Treasurer, Charles W. Hooker.

It was voted to hold the next meeting of the Board at noon on April 17, 1949.

The meeting was adjourned at 10:15 P.M.

CHARLES HUGGINS
Chairman, Board of Directors
CHARLES W. HOOKER
Secretary

MINUTES OF THE MEETING OF THE MEMBERS
HELD APRIL 16, 1949

The meeting was held in the Coral Room of the Hotel Fort Shelby and was called to order at 1:48 P.M.

Reading of the minutes of the last meeting was omitted by vote of the members.

The Treasurer's report and the report of the Auditor were read and accepted.

Dr. E. V. Cowdry, Chairman of the Committee on Local Sections, reported the work of his committee and submitted the Resolution regarding the establishment of Sections of the Association recorded in the minutes of the meeting of the Board of Directors on April 15, 1949. It was voted to adopt the resolution.

The President reported the transfer of ownership of

CANCER RESEARCH to the Association as given in the minutes of the meeting of the Board of Directors on April 15, 1949.

The proposal of the Board of Managers of CANCER RESEARCH regarding increasing the dues of active members and the approval of the Board of Directors were reported. After a spirited and general discussion it was voted that "The annual dues of active members be increased to ten dollars and that active membership carry with it a subscription to CANCER RESEARCH."

The President announced the results of the voting for new members of the Board of Directors and declared the new Directors to serve until 1952 to be Drs. J. C. Aub, E. V. Cowdry, E. A. Doisy, and W. U. Gardner.

The President also reported the action of the Board with respect to the order of names on the ballot and announced the decision of the Board regarding a member of the Board succeeding himself. These actions were approved.

The nominations for officers of the Association were read:

Joseph C. Aub, *President*
Jacob Furth, *Vice-President*
Charles W. Hooker, *Secretary-Treasurer*

The candidates were elected.

The conflicts of the meeting with other meetings of interest to members of the Association were pointed out. Dr. Frank B. Queen presented the resolution: "That the Board of Directors of the Association be requested to explore the possibility of coordinating the time and the place, and if not the place, the time, of other meetings with the Executive Boards of the American Association of Pathologists and Bacteriologists, the International Association of Medical Museums, and the American Society for Experimental Pathology; that it is the sense of the members of the Association that these meetings be so timed that the meetings of the several societies will not be mutually exclusive to their members." In the discussion the reason for the present custom of meeting in conjunction with the Federation and the belief that a majority of the Association's members are interested in the Federation's meeting were mentioned. Dr. M. J. Shear pointed out the difficulties experienced by employees of the Federal Government in getting numerous or closely spaced leaves of absence. When submitted to vote, Doctor Queen's resolution was adopted.

The desirability of having the abstracts printed prior to the meeting was pointed out, and the question was briefly discussed without decision.

The meeting was adjourned at 2:25 P.M.

CHARLES HUGGINS
President
CHARLES W. HOOKER
Secretary

MINUTES OF THE MEETING OF THE BOARD OF
DIRECTORS HELD APRIL 17, 1949

The meeting was called to order at 1:15 P.M. in the Hotel Fort Shelby in Detroit, Michigan, following waiver of previous formal notice of the meeting signed

by all Directors present and constituting a quorum. Board members J. J. Bittner, A. M. Brues, E. V. Cowdry, E. A. Doisy, Jacob Furth, W. U. Gardner, C. W. Hooker, and Charles Huggins were present with the new Chairman of the Board, Dr. J. C. Aub, presiding.

Reading of the minutes of the last meeting was waived.

The problem of the relations of the Association with the press was discussed. Dr. Cowdry, who represented the Association in its relations with the press during the present meeting, reported that the Chairmen of the several scientific sessions had met with representatives of the press. It seemed agreed that public interest in the cancer problem and public support of cancer research more or less dictate cooperation with the press, and that the seriousness of the cancer problem makes it imperative that press reports not be misleading, however unintentionally. No ready solution was apparent, but it seemed agreed that if abstracts were available, science writers could come to the meeting better prepared to report papers presented. The advantages of prior publication of abstracts to members of the Association attending the meeting were also stressed. It was voted, "That abstracts of the papers on the program be published in the number of *CANCER RESEARCH* immediately preceding the meeting."

The problem of financing *CANCER RESEARCH* was again discussed, with particular attention to applications to various Foundations for support. It was voted, "That the Board of Directors seek any support that can be obtained without restrictions as to use."

The selection of a new Editor-in-Chief and the sacrifices an Editor must make were discussed. Several competent and desirable candidates were suggested, and means of making the post attractive were considered. The President was directed to proceed toward the selection of an Editor.

It was voted, "That the Executive Council take the necessary steps to transfer the funds of Cancer Research, Inc. to such place as they deem fit."

It was pointed out that the automatic subscription of members and the handling of non-member subscriptions by the publisher have rendered the post of Business Manager of *CANCER RESEARCH* unnecessary. It

was therefore voted, "That the post of Business Manager of *CANCER RESEARCH* be discontinued; that the Business Manager be discharged with the thanks of the President and the Association; that the Secretary-Treasurer of the Association assume the residual duties of the Business Manager; and that the sum of \$1200 be allocated for the office of Secretary-Treasurer." This sum represents \$900 previously allocated by *CANCER RESEARCH* to the office of the Business Manager and \$300 usually appropriated by the Association for the office of the Secretary-Treasurer.

The Chairman of the Board proposed the following as members of the standing committees of the Association:

Program Committee.—A. M. Brues, Chairman; C. P. Rhoads; H. P. Rusch.

Nominating Committee.—C. W. Hooker, Chairman; H. B. Andervont; E. V. Cowdry.

Membership Committee.—Jacob Furth, Chairman; W. L. Simpson; E. C. Reifenstein, Jr.

Committee on Cancer Research, Its Organization and Support.—Shields Warren, Chairman; G. M. Smith.

He also proposed the following Special Committees:

Committee on Press Relations.—E. V. Cowdry.

Committee on Amending By-Laws.—C. W. Hooker, Chairman; Jacob Furth.

The Board approved the proposed Committees.

The customary banking resolutions were adopted.

Publication of the minutes and scientific proceedings of the meeting were authorized, the costs of publication to be paid by the Association.

It was voted, "That publication of the By-Laws and list of members be deferred until after the next annual meeting." This action was prompted by the proposal that the By-Laws be revised and brought to date before the next meeting.

The meeting was adjourned at 2:00 p.m.

JOSEPH C. AUB

Chairman, Board of Directors

CHARLES W. HOOKER

Secretary

CANCER RESEARCH

VOLUME 9

NOVEMBER 1949

NUMBER 11

The Oxidation of Octanoate by Liver Homogenates from Leucemic Mice*

CARL S. VESTLING, JESSE N. WILLIAMS, JR., † SEYMOUR KAUFMAN, ‡
RICHARD E. MAXWELL, § AND HENRY QUASTLER

(From the Division of Biochemistry, Noyes Laboratory of Chemistry, University of Illinois, Urbana, and
the Department of Radiology, Carle Hospital Clinic, Urbana, Ill.)

We have begun an investigation of the level of enzymatic activity in tissues which have been infiltrated by malignant cells. Two preliminary reports of this work have appeared (1, 2). We are presenting herewith results of studies of fatty acid oxidase activity which have been carried out on C58 mouse liver homogenates according to Lehninger (3, 4). In addition, results of orienting experiments with rat liver systems will be reported.

Since one aspect of the cancer problem is the study of the chemical reactions which take place in living tissue, and since all major reactions *in vivo* involve enzymatic influence, it is important to determine the effect which invading malignant cells may have on the catalytic apparatus of normal tissue. The majority of the studies of enzymes in relation to cancer have been similar to those described by Greenstein and co-workers (5) and have involved a comparison of the enzymatic levels of normal *versus* tumor tissue. It seems important to us to study organs which are more or less diffusely infiltrated by malignant cells. The C58 strain of mice developed by MacDowell offers excellent ex-

perimental material for this type of study, since numerous tissues in these animals are invaded by malignant lymphocytes as spontaneous lymphatic leucemia develops (6, 7). Histological examination of typical tissues from C58 mice shows general infiltration with pronounced concentrations of malignant cells around the periphery of blood vessels. Entire organs are attacked and are subjected to a competition for metabolites with the actively multiplying lymphocytes.

Using liver slices from leucemic mice (Rf and Rf/Ak strains) Burk *et al.* (8) have described experiments involving oxygen uptake, CO₂ production, and aerobic and anaerobic glycolysis. Their results show, in general, elevated glycolysis (particularly in anaerobic systems) in the case of infiltrated livers. Hall (9) has reported similar results in anaerobic glycolysis studies on liver slices from leucemic Ak mice. We have confirmed these observations on anaerobic glycolysis with C58 mouse liver slices (10). (For results of studies of the metabolism of lymph nodes from leucemic mice, see Victor and Potter (11).)

Our results can be summarized in advance by stating that in C58 mouse livers there is frequently observed a profound loss of fatty acid oxidase activity (as measured by the Lehninger technique) associated with leucemic infiltration. In such cases the extent of loss of activity usually greatly exceeds the extent of replacement of liver cells by malignant lymphocytes. Histological examination of the C58 mouse livers has been made in every case, and an estimate of the degree of infiltration obtained using Chalkley's method (12). In our experiments whole homogenates and washed, mal-

* The authors wish to thank Dr. E. C. MacDowell, Department of Genetics, Carnegie Institution of Washington, Cold Spring Harbor, Long Island, New York, for supplying them with mice of the valuable C58 strain for these studies.

† In part from a thesis for the degree of Doctor of Philosophy in Chemistry submitted by J. N. Williams, Jr., to the Graduate College, University of Illinois. Present address, Department of Biochemistry, University of Wisconsin, Madison, Wisconsin.

‡ Present address, Department of Biochemistry, School of Medicine, Duke University, Durham, North Carolina.

§ Present address, Department of Chemistry, Iowa State College, Ames, Iowa.

onate-inhibited systems from infiltrated mouse livers showed essentially similar net fatty acid oxidase activities.

Lehnninger (4) reported that rat liver fatty acid oxidase systems were susceptible to inhibition by excess substrate. We have made a study of the relationship between octanoate concentration and oxygen uptake, using whole rat liver homogenates and washed, malonate-inhibited rat liver homogenates. Both types of system show a peak of activity at 0.001 M octanoate concentration. Our mouse experiments have been carried out at this concentration.

EXPERIMENTAL

For the work to be described the following special materials were obtained as indicated: Cytochrome-c by the method of Keilin and Hartree (13) modified by final dialysis against distilled

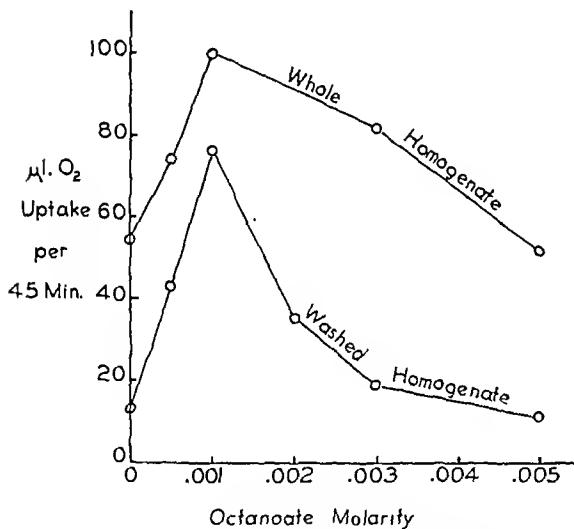


FIG. 1.—The Relation Between Oxygen Uptake and Octanoate Concentration in Rat Liver Homogenates. Reaction Time = 45 minutes. Temperature = 25° C. Flask Components and Reaction Conditions in the "Unwashed" Series:

- 0.20 ml. 0.035 M Na₂HPO₄ (pH = 7.5)
- 0.10 ml. 1 × 10⁻⁴ M cytochrome c
- 0.25 ml. 0.013 M Na₄ATP
- 0.25 ml. unwashed liver homogenate (See text)
- 0.20 ml. 10 per cent KOH in center well
- 0.20 ml. sodium octanoate (pH 7.5) of appropriate concentration or 0.20 ml. H₂O in side-arm.

Flask Components and Reaction Conditions in the "Washed" Series:

- 0.10 ml. 0.07 M Na₂HPO₄ (pH = 7.5)
- 0.10 ml. 0.048 M MgSO₄
- 0.10 ml. 1 × 10⁻⁴ M cytochrome c
- 0.10 ml. 0.1 M Na malonate
- 0.10 ml. 0.033 M Na₄ATP
- 0.20 ml. KOH in center well
- 0.30 ml. homogenate (washed)
- 0.20 ml. sodium octanoate (pH 7.5) of appropriate concentration or 0.20 ml. H₂O in side-arm

water and lyophilization, adenosine triphosphate according to LePage (14), octanoic acid (Eastman), α -ketoglutaric acid synthesized by E. D. Nielson in this laboratory according to Schneider (15), adenylic acid by hydrolysis of adenosine triphosphate.

Livers were homogenized for 2 to 3 minutes in two parts of ice-cold Krebs-Ringer phosphate buffer (pH = 7.7; calcium omitted) with the aid of a Potter-Elvehjem glass homogenizer (16). After removal from the animals the livers were immediately cooled in ice for 5 minutes before homogenizing. In the preparation of washed systems the whole homogenates were centrifuged at 0 to 5°

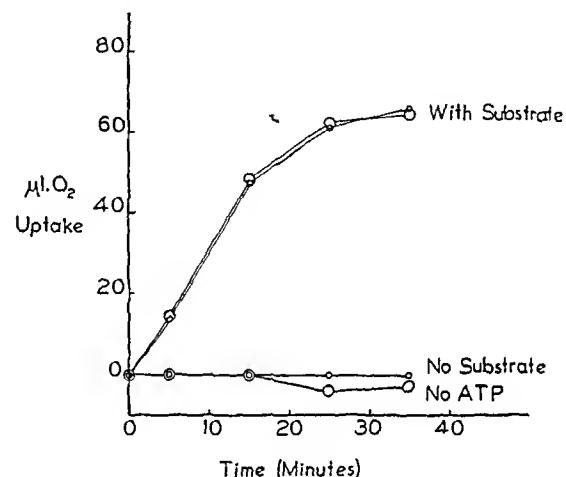


FIG. 2.—Oxidation of Octanoate by C58 Mouse Liver Homogenate (Washed, Malonate-inhibited). Mouse #77293. Per Cent Infiltration of Liver = 0.7. Theoretical Oxygen Uptake = 67.2 μ l. Temperature = 25° C. Flask Components and Reaction Conditions:

- 0.10 ml. 0.025 M Na₂HPO₄ (pH = 7.5)
- 0.10 ml. 0.083 M sodium malonate
- 0.10 ml. 0.048 M MgSO₄
- 0.10 ml. 1 × 10⁻⁴ M cytochrome c
- 0.15 ml. 0.022 M Na₄ATP
- 0.25 ml. washed liver homogenate (See text)
- 0.20 ml. 10 per cent KOH in center well
- 0.20 ml. sodium octanoate (pH 7.5) of appropriate concentration or 0.20 ml. H₂O in side-arm

for 5 minutes in a clinical centrifuge at 2500 r.p.m. and the supernatant fluid discarded. The centrifuged material was then washed with ice-cold Krebs-Ringer phosphate buffer in sufficient amount to bring the system to its original volume after centrifugation. The washing and centrifuging were done 3 times. After the final washing the system was made up to three-fourths of its original volume with the ice-cold buffer.

In our early experiments Lehninger's first technique (3) involving whole homogenates was used (17). It became apparent that further knowledge of the effects of varying substrate concentration was required. Hence, the oxygen uptake of rat liver systems as a function of octanoate concentration was studied. The results of an

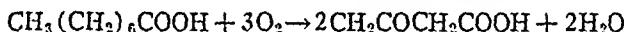
experiment of this type are shown in Figure 1. On this graph are also plotted results for a washed malonate-inhibited rat liver system. These results indicate that 0.001 M octanoate concentration permits optimal activity.

From experiments with washed, malonate-inhibited rat liver systems it was possible to plot the reciprocals of the initial velocities (15 minutes values of the oxygen uptake) against the reciprocals of the octanoate concentrations. This permits evaluation of the Michaelis constant (K_m) according to the slope-intercept form of the Michaelis equation: (18)

$$\frac{1}{v} = \frac{K_m}{V_{max} \cdot S} + \frac{1}{V_{max}}$$

In this equation v is the observed initial velocity, V_{max} is the graphically estimated limiting velocity, and S is substrate molarity. The values of K_m for 6 experiments with normal adult rat liver were 1.88, 1.64, 2.20, 1.71, 1.31, and $1.43 \times 10^{-3} M$. The average K_m value was $1.70 \times 10^{-3} M$.

Lehninger's (19) and our results with the washed systems show a rapid near-quantitative transformation of octanoate to acetoacetate under the conditions specified in Figure 1. The oxygen uptake approaches the theoretical amount required for the reaction:



Chemical analysis for acetoacetic acid produced in the reaction by Edson's method (25) amply confirms Lehninger's results. An indication of the reproducibility of the results encountered in the rat liver systems may be obtained from the following: Experiments with 15 normal rat livers from stock animals of various ages and both sexes showed an average oxygen uptake in 45 minutes of $48 \pm 8 \mu\text{l}$. This corresponds to 72 ± 12 per cent of the theoretical activity and was checked in several cases by acetoacetate analysis.

Essentially similar results were obtained with C58 mouse liver systems prepared from animals in which there was very little or no infiltration by malignant lymphocytes. In Figure 2 are presented the results of a typical experiment with a non-leucemic (or pre-leucemic) C58 animal. Similar activities were encountered with the livers from mice of other strains (C57 black and Zr).¹ The average oxygen uptake for two C57 animals and one Zr animal studied according to these exact conditions was $53 \pm 7 \mu\text{l}$. in 45 minutes.

It should be emphasized at this point that a major factor involved in the reproducibility of re-

sults appears to be homogenization with a rather loose-fitting homogenizer for short periods of time. We adopted a rigid time schedule of operations in handling the livers. Whenever a system of low activity was encountered, a normal rat liver was tested with the same reagents in order to make sure that all solutions were properly constituted.

In Figure 3 are shown the results of an experiment with a severely infiltrated C58 mouse liver. In this case there was substantially no fatty acid oxidase activity. Other leucemic animals showed intermediate levels of oxidase activity.

In Figure 4 are presented data from experiments designed to examine the relation between oxygen uptake and octanoate concentration in the case of three C58 mice: one a non-leucemic animal and the other two decidedly leucemic. It is apparent from these and similar experiments that 0.001 M octanoate is a favorable concentration for a comparison between non-infiltrated and infiltrated livers. It was not possible to obtain reliable estimates of the Michaelis constant in the case of severely infiltrated mouse livers.

The results of fatty acid oxidase studies on thirty-two C58 mice are summarized in Table 1. In this table are included data from experiments with

washed, malonate-inhibited systems only. Substantially similar results were obtained with whole homogenates. The per cent conversion of octanoate to acetoacetate was calculated from the oxygen uptake after 35 minutes in each case, at which time the systems had practically stopped absorbing oxygen. The mouse numbers are those of MacDowell.

The per cent infiltration values must be regarded as estimates only. Two randomly selected pieces of liver from each animal were removed when the animal was sacrificed. These were fixed in alcohol-formalin-acetic acid, imbedded in paraffin, sectioned and stained with eosin-hematoxylin.² The sections were evaluated using Chalkley's (12) technique: We have as a rule examined 20 fields using an ocular equipped with 5 pointers, and have found that the results are sufficiently accurate for our purposes, and quite well reproduced in independent counts made by different observers. In this way the numbers recorded in Table 1 were obtained. We feel that we have arrived at reasonable approximations of the extent of infiltration, even though in some animals the infiltration was rather spotty.

¹ The authors wish to thank Dr. Herman B. Chase, of the Department of Biology, Brown University, Providence, Rhode Island, for the C57 and Zr mice used in this work.

² The authors wish to thank Dr. Eldon D. Nelson and Ursula Irish for valuable assistance with the histological work.

There is a good correlation of spleen size with the severity of the leucemic state of C58 animals (Table I). We have weighed the spleens in every case and considered spleens weighing less than 0.2 gms. to be normal or pre-leucemic. As described by MacDowell, the leucemic C58 animal frequently exhibits spectacular enlargement of liver, thymus, spleen and lymph nodes. In a few cases fatty livers were found, and in all of these cases fatty acid oxi-

retical oxidase activity. Of the 13 mice exhibiting more than 50 per cent of the theoretical fatty acid oxidase activity, 10 fall in the range, 80 to 100 per cent activity, and of these only one (8 per cent infiltration) showed an infiltration by malignant cells greater than 3 per cent. Thus it appears that certain of these animals show effects of leucemic infiltration to a greater extent than others. Some of the variability in our results is considered to be due to the fact that we were encountering the consequences of malignant cell invasions of widely varying duration. Variations in results from mice with low degrees of liver infiltration also suggest the possibility that the severity of the leucemic state may not always be accurately indicated by

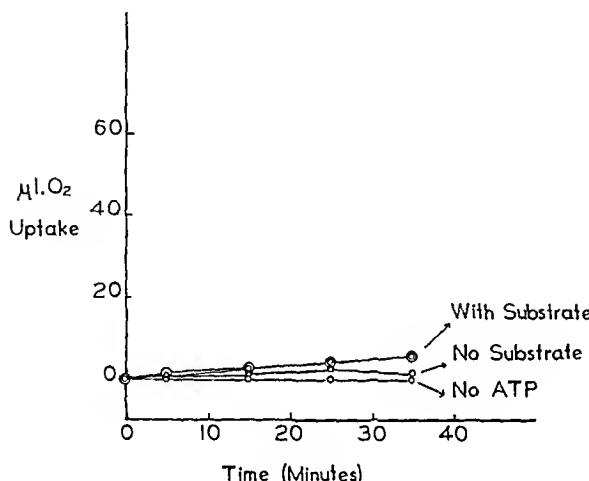


FIG. 3.—Oxidation of Octanoate by Leucemic C58 Mouse Liver Homogenate (Washed, Malonate-inhibited). Mouse #79869. Per Cent Infiltration of Liver = 33. Theoretical Oxygen Uptake = 67.2 μ l. Temperature = 25° C. Flask Components and Reaction Conditions:

- 0.10 ml. 0.025 M Na₂HPO₄ (pH = 7.5)
- 0.10 ml. 0.083 M sodium malonate
- 0.10 ml. 0.048 M MgSO₄
- 0.10 ml. 1 \times 10⁻⁴ M cytochrome c
- 0.15 ml. 0.022 M Na₃ATP
- 0.25 ml. washed liver homogenate (See text)
- 0.20 ml. 10 per cent KOH in center well
- 0.20 ml. sodium octanoate (pH = 7.5) of appropriate concentration or 0.20 ml. H₂O in side-arm

dase activity was very low. A few of the leucemic animals were severely emaciated with almost complete loss of body fat. Several of these animals showed fatty livers. The majority of the leucemic animals possessed reasonably active appetites at the time of sacrifice, so that our results cannot be ascribed to inanition (20).

From the results in Table I we conclude that there is often a failure of systems from infiltrated livers to attack octanoate. Inspection of the data obtained from 32 animals of varying leucemic state reveals that 19 of them showed less than 50 per cent of the theoretical fatty acid oxidase activity. Of these 19, only 5 animals showed malignant cell infiltration higher than 10 per cent, and 13 animals showed less than 20 per cent of the the-

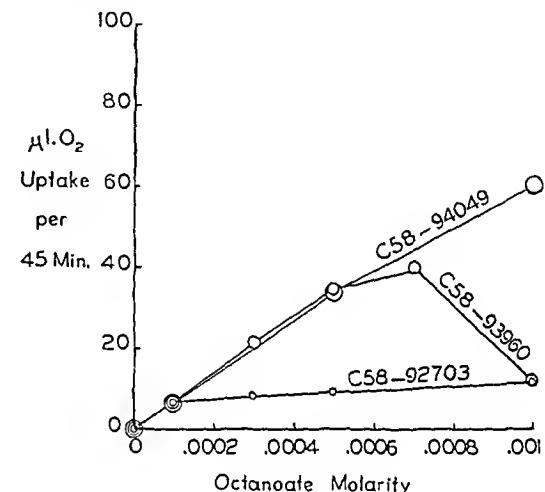


FIG. 4.—The Relation Between Oxygen Uptake and Octanoate Concentration in Washed, Malonate-inhibited C58 Mouse Liver Homogenates. Mouse #94049, Per Cent Infiltration of Liver = 0; Mouse #93360, Per Cent Infiltration = 5.0; Mouse #92703, Per Cent Infiltration = 5.0. Theoretical Oxygen Uptake corresponding to 0.001 M. Octanoate = 67.2 μ l. Temperature = 25° C. Flask Components and Reaction Conditions:

- 0.10 ml. 0.025 M Na₂HPO₄ (pH = 7.5)
- 0.10 ml. 0.083 M sodium malonate
- 0.10 ml. 0.048 M MgSO₄
- 0.10 ml. 1 \times 10⁻⁴ M cytochrome c
- 0.15 ml. 0.022 M Na₃ATP
- 0.25 ml. washed liver homogenate (See text)
- 0.20 ml. 10 per cent KOH in center well
- 0.20 ml. sodium octanoate (pH = 7.5) of appropriate concentration or 0.20 ml. H₂O in side-arm

the extent of malignant cell invasion. In the case of No. 82646 a low infiltration was associated with a large spleen. Animals 93501, 79365, 93529 and 75779 showed somewhat enlarged spleens, but small amount of infiltration and substantially normal fatty acid oxidase activity. The significance of the grossly fatty livers encountered in several animals is not understood.

DISCUSSION

Since fatty acid oxidase activity is known to be associated with the sedimenting particles in a liver homogenate (21, 22), it seemed of interest to compare systems homogenized in Krebs-Ringer phosphate with those homogenized in hypertonic 0.88 M sucrose, which medium tends to preserve particulate structure. No difference in fatty acid oxidase performance was observed in the case of a severely infiltrated mouse liver when it was homogenized in either medium. Potter (23) has shown that homogenization of rat liver in hypotonic medium leads to loss of fatty acid oxidase activity. This loss may be considered to be associated with the disorganization of certain subcellular elements. Potter (24) has suggested that the "cytolysis quotient" is an index to the integrity of certain intracellular entities. The term, "cytochrome quotient," might be applied to suggest the possibility that homogenizing in hypotonic medium leads to

TABLE I
OXIDATION OF OCTANOATE TO ACETOACETATE
BY WASHED, MALONATE-INHIBITED C58
MOUSE LIVER HOMOGENATES†‡

MOUSE NUMBER	PER CENT OF THEORETICAL OXYGEN UPTAKE AFTER 35 MINUTES	PER CENT INFILTRATION OF LIVER	WEIGHT OF SPLEEN (IN GM.)
			OR
91019	84.0	0	0.10
92657	94.0	0.1	0.15
92656	31.0	0.1	0.15
82616 (Male)‡	5.4	0.3	0.60
82529 (Male)	15.0	0.5	0.15
73348	28.8	0.5	0.35
77293	97.5	0.7	0.15
75648	23.6	1.0	0.65
93961	65.5	1.0	0.15
91537	98.0	1.0	0.20
79866	81.5	1.2	0.30
93501	100.0	2.0	0.25
93517	67.0	2.0	0.15
93520	73.0	2.0	0.45
93532	10.0	2.0	0.25
79365	91.5	2.0	0.40
73583	23.8	3.0	0.60
75750	10.9	3.0	0.90
75055	1.5	3.0	2.60
93539	99.0	3.0	0.25
73749	53.5	3.5	0.18
93960	18.8	5.0	0.18
92703	18.2	5.0	0.55
84277‡	7.2	7.0	0.40
73779	91.0	8.0	0.40
93317	0.0	9.0	0.30
75056	6.7	10.0	0.70
79869‡	5.8	33.0	1.20
93318‡	23.2	56.0	1.20
93153	20.2	49.0	1.45
92445	5.0	70.0	1.30
93945‡	5.0	78.0	1.50

* Females used except where otherwise noted.

† Theoretical oxygen uptake = 67.2 μ l.

‡ Grade 5 fatty liver.

‡ Exact Mac-Dowell number unavailable.

the dissociation of cytochrome from certain protein surfaces. In our studies homogenization of livers from a series of mice of varying leucemic state showed no change of "cytochrome quotient" as a function of degree of infiltration (10). Accordingly our fatty acid oxidase results are not readily explained on the basis of fragility of intra-cellular elements, but seem to suggest an actual deficiency of enzymatic surface.

In a few experiments with C58 mice α -ketoglutarate and adenylic acid were substituted for ATP (4). Similar results with infiltrated livers were obtained when this modification was employed.

In one experiment equal amounts of washed normal mouse liver (Zr strain) and of highly infiltrated C58 mouse liver were used in an attempt to detect any possible inhibitor effect. No such effect was observed. The Zr mouse liver system and the mixed system showed a near-quantitative conversion of octanoate to acetoacetate, but the C58 leucemic mouse liver system was almost wholly inactive.

An additional point needs to be made. Since we are studying the enzymatic activity of liver "systems" of variable malignant lymphocyte content, we may get a picture of some of the enzymatic capabilities of lymphocytes in cases of severe infiltration. However, in those cases where the infiltration is low, it is our opinion that we are dealing with lowered activity of the liver cells themselves.

How far these results may be considered to bear upon the situation existing in the intact animals is difficult to state. We feel that the best interpretation of the data at present is based upon the concept of a competition for metabolites in an invaded organ between the rapidly multiplying malignant lymphocytes and the liver cells. This competition favors the lymphocytes, in part because of their concentration around the periphery of blood vessels. The net result is a deficiency of certain intracellular proteins (enzymes). In support of this idea is the fact that our studies showed in several cases a sharp loss of fatty acid oxidase activity in livers which were infiltrated to only a small extent (less than 10 per cent).

SUMMARY

1. The oxidation of octanoate in normal rat liver and in C58 mouse liver homogenates has been studied.

2. Data from thirty-two C58 animals of varying leucemic state have been presented. Nineteen of these animals showed fatty acid oxidase activi-

ties less than 50 per cent of the theoretical. Thirteen of these 19 animals yielded homogenates exhibiting less than 20 per cent of the calculated activity.

3. A discussion of possible implications of these results is presented.

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Free Amino Acids in Normal and Neoplastic Tissues of Mice as Studied by Paper Chromatography*†

EUGENE ROBERTS AND SAM FRANKEL

(From the Division of Cancer Research, Department of Anatomy, Washington University School of Medicine,
St. Louis, Missouri)

Changes found in the free amino acid content of epidermis undergoing carcinogenesis as determined by two-dimensional paper chromatography have been reported recently from this laboratory (1). The present paper deals with the determination of the patterns of free amino acids in a variety of mouse tumors, comparing the neoplasms in as many cases as possible with the normal homologous tissues. Comparisons were made of muscle with rhabdomyosarcoma, liver with hepatoma, epidermis with squamous cell carcinoma, and lymph nodes with lymphosarcoma. In addition, chromatograms were made of extracts of testes and interstitial cell tumor of the testes. No direct comparison is possible in the latter case because only a small portion of the cells of the testes is of the interstitial type. Samples of a number of other tumors were also examined for which no suitable comparison could be made with a normal tissue. The results obtained for a large number of normal tissues not discussed herein will be reported separately.

MATERIALS AND METHODS

The transplantable lymphosarcoma and interstitial cell tumors of the testes which were carried in F₁ hybrid mice of C57 ♀ × CBA ♂ and C3H; ♀ × Ao ♂, respectively, were furnished by Dr. W. U. Gardner of Yale. Samples of a rapidly growing transplantable hepatoma in the leaden strain were received from the Jackson Memorial Laboratories and a transplantable rhabdomyosarcoma in the C3H strain was obtained from Dr. Elizabeth Green of the Institute for Cancer Research in Philadelphia. Sarcoma 180 in Carworth Farms CFW male mice, sarcoma T 241 in C57 black male mice, and tumor MA 387 in AKR male mice were furnished by Dr. J. Biesele of the Sloan-Kettering Institute. Samples of sarcoma 37 were obtained from Dr. Virginia Downing of the National Cancer Institute and Dr. F. A. Jacobs of St. Louis University. Transplantable squamous cell carcinomas carried in Swiss mice in this labor-

ratory (2) and spontaneous mammary carcinomas arising in Swiss female mice in our colony were also studied.

Determinations were made by the two-dimensional chromatographic method (3, 4, 5). Samples were prepared with and without H₂O₂. Treatment with H₂O₂ is necessary for the detection of cystine as cysteic acid and methionine as methionine sulfone. Many of the chromatograms were run in duplicate or triplicate, aliquots of the same sample invariably showing the same patterns. Different preparations of an organ from animals of a given species exhibited remarkable constancy in the proportions of free amino acids.

Fresh tissues, freed of visibly detectable necrotic material, were weighed rapidly on a torsion balance after excision and immediately placed in ethyl alcohol, the final concentration of alcohol ranging between 70 and 73 per cent. No differences were observed when the tissues were frozen in dry ice immediately after removal. The material was homogenized in a ground-glass homogenizer, centrifuged, and the supernatant fluid was decanted and evaporated to dryness. The residue was then thoroughly extracted with distilled water (1 ml. per gm. of fresh weight of tissue). Aliquots corresponding to 75 mg. of fresh weight of tissue were placed on paper for chromatography. Results identical with those previously reported for extracts of rapidly dried samples of epidermis and squamous cell carcinoma (1) were obtained when the above procedure was applied to these tissues. It was found that dialysates, obtained when homogenates of heat-coagulated tissues were equilibrated with a small quantity of distilled water in a rocking dialyzer (6), gave the same patterns as did the alcoholic extracts. The latter method is preferable when an estimation of the amino acids present in peptide or polypeptide form is desired, since small amounts of alcohol-soluble proteins which do not interfere with the satisfactory resolution of amino acids on the chromatograms would give significant quantities of amino acids after acid or alkaline hydrolysis.

The amino acids were identified by their relative positions on the paper square and by comparison with a reference chromatogram prepared by Dent (7). The positions of individual amino acids were checked from time to time by the addition of known amino acids to tissue extracts prior to running the chromatograms. At all times the added amino acids migrated to the expected positions. The failure to detect the presence of an amino acid was taken to indicate that the quantity of the ami-

* Aided by grants from the U. S. Public Health Service and the Charles F. Kettering Foundation.

† Presented at the 10th Meeting of the American Association for Cancer Research, Detroit, April 17, 1949.

no acid present was below the level detectable under the conditions employed and not necessarily that the amino acid was completely absent from the solution tested. The aliquots used in this study were chosen so that in most cases the amino acids occurring in greatest amounts did not give spots which were too intense to allow a semi-quantitative estimate of the amount present.

RESULTS

General comment.—A diagram showing the positions of the ninhydrin-reactive substances as they were distributed on our chromatograms is given in Figure 1. Spot X was given by glutathione either in pure solution or on addition to a casein digest. On treatment of either solution with H_2O_2 , this spot is shifted to position V. This spot disappears after

of the isolated material. On subsequent chromatography of an acid hydrolysate of an identical aliquot of the material, the spot corresponding to W was found to have disappeared. Spots corresponding to glycine, alanine, the leucines, serine, threonine, proline, and cysteic acid appeared, and there was an increase in the quantity of glutamic acid and a possible small increase in the quantity of aspartic acid. From these results "underglutamic acid" isolated from squamous cell carcinoma would appear to be a polypeptide containing at least 9 or 10 amino acids. Further studies are being made on the isolation, purification, and identification of this material from various sources.

A. high level of taurine was found in extracts of all of the mouse tissues examined, both normal and neoplastic. Somewhat smaller quantities were found in human tissues. The physiological significance of the presence of this substance in large quantities in various tissues is not known. Dent has reported that taurine may be increased in the urine in pathological states (7). A decrease in the taurine level of mouse epidermis was produced by the application of pure benzene (1). The ninhydrin- CO_2 method for the determination of amino acids does not determine taurine. Therefore studies on tissue extracts employing this method would not detect variations in taurine which might be important in pathological states and during the active uptake of amino acids by a tissue.

Free amino acids in tumors and normal homologous tissues.—Photographs of typical chromatograms are shown in Figures 2 through 13. The yellow spot given by proline is difficult to detect on the photographs because there is insufficient contrast, but is easily visible on the original chromatograms. The neoplastic tissues were compared with the normal homologous tissue from the animals bearing the tumor. Patterns of free amino acids in non-cancerous tissues of a tumor-bearing mouse were not found to be significantly different from those in normal controls, when it was possible to make the comparison in animals of the same age and strain.

Normal mouse epidermis has an extremely high content of free amino acids (Fig. 2) (1). This is consonant with the extremely high level of non-protein nitrogen found in this tissue (9). The squamous cell carcinomas (Fig. 3) showed a striking decrease in the quantity of detectable constituents on the chromatograms in agreement with the markedly decreased content of non-protein nitrogen (9). The "underglutamie acid," cystine, and taurine spots had a greater intensity in the tumors than in the normal epidermis. Glutamine fell from

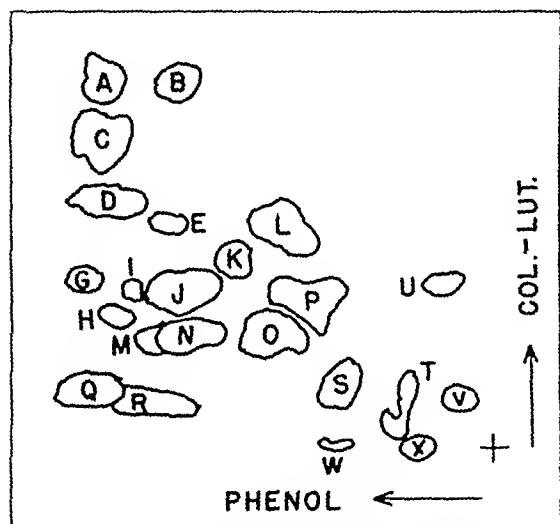


FIG. 1.—Diagram of constituents of chromatograms: A, phenylalanine; B, tyrosine; C, leucine; D, valine; E, methionine sulfone; G, proline; H, histidine; I, hydroxyproline; J, alanine; K, threonine; L, taurine; M, β -alanine or eitrulline; N, glutamine; O, glycine; P, serine; Q, arginine; R, lysine; S, glutamic acid; T, aspartic acid; U, cysteic acid; V, "oxidized" glutathione; W, "underglutamic acid"; X, glutathione.

acid hydrolysis and equivalent quantities of glutamic acid, glycine, and cysteine appear. Spot W, which has been designated "underglutamie acid" (1), has been detected in extracts of mouse, human, and frog tissues and in plants and bacteria, but not in human or mouse plasma. It was found to disappear on acid hydrolysis. The material corresponding to this spot was isolated from carcinoma extracts by the one-dimensional technique in collidine-lutidine as described by Dent (4), and used by Tishkoff *et al.* (8) for the fractionation of liver extract. Only glutamie and aspartic acids were found as contaminants on subsequent two-dimensional chromatography of an aliquot of a solution

a high level in the normal epidermis to a level not detectable in the tumors. Although the proline level fell markedly in the tumors, easily detectable quantities were still present. A comparison of chromatograms made from squamous cell carcinomas with necrotic material obtained therefrom showed clearly that the pattern of free amino acids in the tumors could not result from contamination with necrotic tissue.

Both the muscle and the rhabdomyosarcoma (Figs. 4 and 5) had high contents of taurine. However, the tumor had a higher content of glutathione, "underglutamic acid," glutamic acid, glycine, alanine, proline, serine, cystine, and the other detectable constituents.

The taurine level was high in both the lymph nodes and the lymphosarcoma (Figs. 6 and 7). The lymph nodes had a considerably higher content of aspartic acid. Approximately the same quantities of glutathione, "underglutamic acid," and glutamic acid were present in both tissues. The lymphosarcoma had a higher level of glycine, alanine, serine, proline, and cystine.

The taurine, alanine, and valine levels in the liver and hepatoma (Figs. 8 and 9) were approximately the same, while the glutamic acid, glycine, serine, proline, aspartic acid, leucine, and cystine levels were higher in the tumor. On the other hand, the glutamine level of the hepatoma was lower than that of the liver, as was the case in the comparison of squamous cell carcinoma with its normal homologue.

Other tissues.—The testes (Fig. 10) showed a pattern of free amino acids which differed from that of the other normal tissues, while the interstitial cell tumor (Fig. 11) had a distribution which was similar to that shown by the other tumors examined. Chromatograms were made of a number of samples of sarcomas 37, 180, and T 241, spontaneous mammary carcinoma, and tumor MA 387. Photographs are shown of chromatograms of samples of sarcoma 37 and MA 387 (Figs. 12 and 13). There were no major differences observed between these neoplasms and those discussed previously. These findings emphasize the similarity of the free amino acid distribution in various neoplastic tissues.

An examination of the photographs of the chromatograms shows clearly that the neoplastic tissues tend to approach the same pattern of free amino acids whether they arose originally from a tissue which had a high content of free amino acids, such as epidermis, or a tissue relatively low in these constituents such as muscle. It is apparent that each of the normal tissues has a characteristic dis-

tribution of free amino acids which is different from that of the other normal tissues, while the patterns of the tumors are similar in most respects to each other.

DISCUSSION

The finding that there is a similar pattern of free amino acids in neoplastic tissues is consistent with the previously reported similarity in enzymic constitution (10) and vitamin content (11), and supports the hypothesis that the metabolism of malignant tumors approaches a common type. The work of Gale with bacteria (12) and Christensen with animal tissues (13 to 17) has proven conclusively that the uptake of amino acids by living cells is a highly selective process and is dependent on an expenditure of energy by the cell. Christensen (17) has recently shown that the maintenance of a higher concentration of amino acids in the cell than in the medium also requires energy. The above-mentioned experiments do not exclude the possibility that the free amino acids normally present in the cells may come, at least in part, from synthesis or from the breakdown of larger molecules such as polypeptides or proteins.

The numerous problems suggested to us by the present study are now under active investigation in this laboratory.

SUMMARY

Patterns of free amino acids determined by two-dimensional paper chromatography were studied in a variety of mouse tumors, comparing the neoplasms in several cases with the normal homologous tissues. It was found that each normal tissue examined had a distribution of free amino acids characteristic for that tissue, while all of the tumors, regardless of derivation or source, showed very similar patterns.

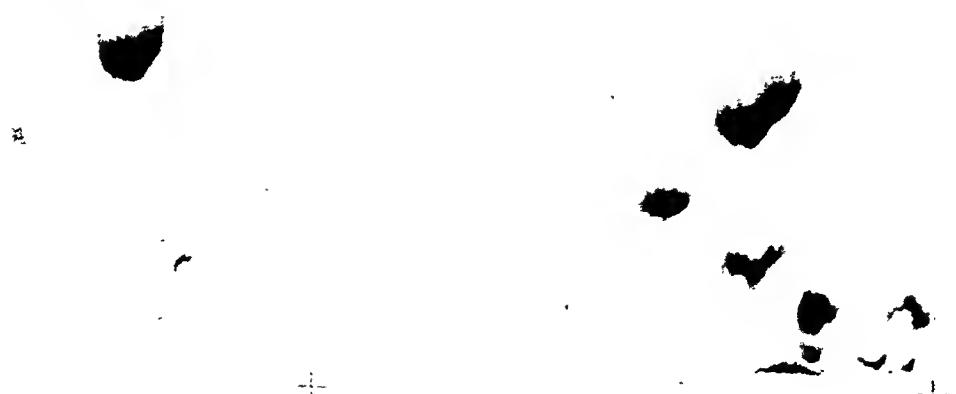
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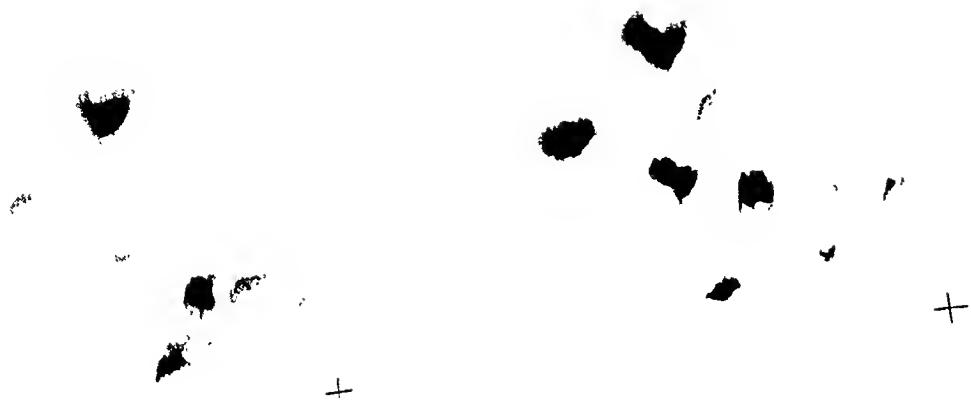


Figs. 2 to 3



Figs. 4 to 5

Figs. 2 to 13.—Peroxide-treated extracts of tissues. Aliquots corresponding to 75 mg. of fresh weight of tissue were employed in each case.



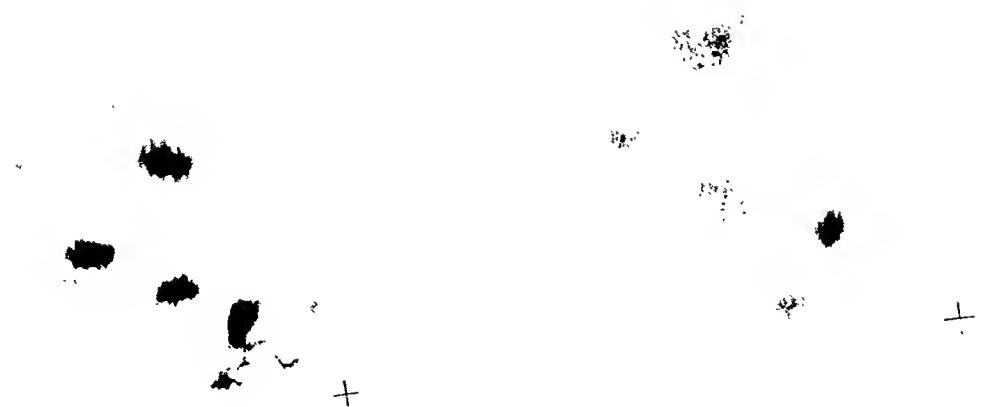
FIGS. 6 TO 7



FIGS. 8 TO 9



FIGS. 10 TO 11



FIGS. 12 TO 13

Factors Affecting the Distribution of Tumor Metastases

Experiments with V₂ Carcinoma of Rabbits*

DALE REX COMAN, M.D., RICHARD B. EISENBERG, M.D., AND
MORTON McCUTCHEON, M.D.

(From the Department of Pathology, University of Pennsylvania School of Medicine, Philadelphia 4, Pennsylvania)

In the study of tumor metastasis, a major problem is to account for the distribution of secondary tumors in different anatomic locations, and especially for the scarcity of metastases in certain organs, notably in the spleen and in the muscles.

One explanation that has been advanced is that some organs may afford a less favorable environment for the growth of embolizing tumor cells than do others. Thus Paget (1), in 1889, likened tumor emboli to "seeds" falling in "soils" of different degrees of fertility, and this concept of the "suitability of the soil" as favoring or inhibiting the establishment of tumor emboli and their development into secondary tumors has been accepted by many writers (2). By "soil" is meant the various chemical and physical factors that may differ from one part of the body to another. Thus in voluntary muscle it is conceivable that production of lactic acid or mechanical stress might be unfavorable to survival and growth of embolizing cells.

Another possible explanation is that metastases may fail to appear in certain organs because tumor emboli fail to reach them.

The present investigation was designed to test the validity of this hypothesis, which we may express as follows: tumor metastasis will develop in any organ that receives viable tumor emboli in sufficient numbers, so that scarcity of metastases in an organ is explainable by scarcity of emboli. This hypothesis may readily be tested by injecting cancer cells into the arterial blood supply of the organ. If, in consequence, metastases regularly develop there, the hypothesis of organ susceptibility or suitable "soil" would, at least for the tumor and species studied, be unnecessary.

MATERIAL AND METHODS

The tumor used in these experiments was a squamous cell carcinoma of rabbits, the V₂ carcinoma derived from the Shope virus papilloma.

* This investigation was supported by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U.S. Public Health Service.

This tumor is readily maintained in domestic rabbits; it metastasizes regularly to adjacent lymph nodes, occasionally to the lungs, rarely elsewhere, in this behavior resembling squamous cell carcinoma in man.

Cancer cells were prepared for injection by passing viable portions of tumor through a fine mesh metal sieve and suspending the cells in 5 volumes of saline (Gey's solution); 0.3 to 1.0 cc. of this suspension was injected intravascularly in rabbits.

RESULTS

Intra-arterial injections.—Since skeletal muscles are seldom the seat of spontaneous metastases, embolism to these organs, if it should regularly result in the development of metastases, would give strong support to the hypothesis being tested. Accordingly, a tumor cell suspension was injected into the femoral artery. In each of 8 rabbits at the time of sacrifice, 65 to 86 days later, there was found extensive infiltration of the leg muscles with confluent masses of neoplasm (Fig. 1). Evidently cancer cells, providing they are able to reach muscle, have no difficulty in establishing metastases there. In other words, there is no indication that muscle provides an unfavorable "soil."

Injections into the left side of the heart.—In order further to test the hypothesis that metastases will develop in any organ receiving an adequate number of tumor emboli, a suspension of tumor cells was injected into the left side of the heart. Cancer cells should in consequence be carried to all organs and metastases should appear everywhere. That is approximately what happened. In Table 1 is shown the distribution of metastases by organs. It is interesting to observe that muscles, skin, myocardium, and lungs head the list in frequency. In each of 6 rabbits, great numbers of secondary tumors appeared in voluntary muscles and skin, as well as in many other organs (Figs. 2 to 3). As shown in Figure 3, metastases were frequent in the musculature of the trunk. In muscles of the extremities, secondary tumors were less common and

occurred in greater numbers in the proximal than in the distal portions. As to visceral metastases, these are shown in Figure 2 in the liver, lungs, spleen, intestine, and kidneys. The histologic appearance of a metastatic tumor nodule in muscle is shown in Figure 4.

Thus it has been found that if cancer cells get into the left side of the heart they produce metastases everywhere. And yet, as mentioned earlier, this tumor rarely metastasizes spontaneously except to the regional lymph nodes and lungs. This is true, obviously, because few cancer cells reach the left side of the heart, having been trapped in the lungs.

Injections into the right side of the heart and into the femoral vein.—The question was now asked: How efficient are the lungs in preventing tumor cells from getting through into the systemic arteries?



FIG. 1.—Hind leg of a rabbit showing massive infiltration of the musculature by neoplastic tissue following injection of a tumor cell suspension into the femoral artery.

TABLE 1

DISTRIBUTION OF METASTASES IN 6 RABBITS
INOCULATED WITH TUMOR CELL SUSPEN-
SIONS INTO THE LEFT SIDE OF THE HEART

Site*	Number of animals in which metastases were observed
Musculature	6
Skin	5
Myocardium	5
Lungs	5
Kidneys	4
Eye (anterior chamber)	3
Liver	2
Pancreas	2
Adrenals	1
Bowel	1
Brain	1
Lymph nodes	1
Spleen	1

* The skeleton was examined in only one animal and was found to contain metastases in several of the long and flat bones.



FIG. 2.—Viscera of a rabbit following injection of a tumor cell suspension into the left side of the heart. Nodules of tumor are seen in the intestine, liver, spleen, kidneys, lungs, and heart.



FIG. 3.—A rabbit with skin reflected to show numerous metastatic nodules in the muscles of the trunk and legs and in the subcutaneous tissue, following injection of a tumor cell suspension into the left side of the heart.

To answer this question a heavy suspension of these cells was injected into the femoral vein or the right side of the heart. In each of 4 rabbits so injected and sacrificed from 35 to 40 days later, innumerable tumor nodules were found in the lungs (Fig. 5) but not a tumor in any other organ. Evidently the cells of this cancer were unable to pass through the lungs, and no secondary metastases

arived from the Shope rabbit papilloma. When suspensions of cells from this tumor were injected into the femoral artery of rabbits, massive neoplastic growths infiltrated the leg muscles, showing that skeletal muscles readily support secondary tumors. If tumor cells are injected into the left side of the heart, they should be distributed to and produce metastases in all organs. This was verified experimentally. Hence scarcity of metastases in an organ is due to scarcity of tumor emboli in the systemic arterial blood, as the result of screening by the lungs. Screening of cells of this tumor was found to be highly efficient, as injection of cancer cells into the femoral vein was followed by a multitude of secondary tumors in the lungs but not one tumor was found elsewhere. It is concluded that

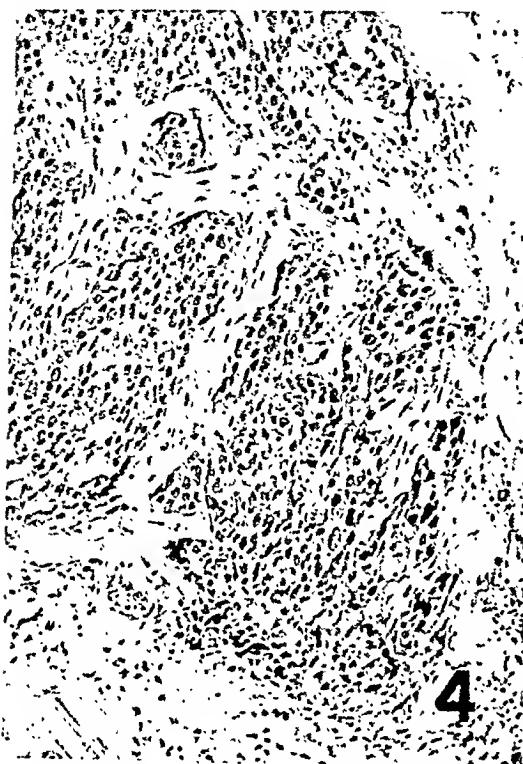


FIG. 4.—Histologic appearance of the tumor as seen in a metastatic nodule in muscle. Mag. X130.

arose from the primary metastases. It follows that when in the presence of a tumor metastasizing to the lungs, metastases do not develop elsewhere, it is because tumor cells do not pass in adequate numbers from the lungs into the systemic arteries.

SUMMARY

Scarcity of tumor metastases in certain organs, such as voluntary muscles, might be due to low tumor susceptibility (unfavorable "soil") or to failure of cancer cells to reach these organs. If the latter explanation is correct, secondary tumors should arise in all organs receiving an adequate number of embolizing tumor cells. This can be accomplished by injecting a suspension of tumor cells into the arterial blood supply of an organ. The tumor employed was the V₂ carcinoma de-



FIG. 5.—Lungs of a rabbit following injections of a tumor cell suspension into the right side of the heart.

scarcity of metastases in an organ is explainable by scarcity of tumor cells reaching that organ. Under the conditions of these experiments, no organ proved to be unfavorable soil.

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The Carcinogenic Activities of Certain Halogen Derivatives of 4-Dimethylaminoazobenzene in the Rat*

J. A. MILLER, PH.D., R. W. SAPP, M.S., AND E. C. MILLER, PH.D.

(From the McArdle Memorial Laboratory, Medical School, University of Wisconsin, Madison 6, Wisconsin)

Previous studies from this laboratory (17, 19) have demonstrated that methyl, chloro, or nitro groups in the 2', 3', or 4' positions of the hepatic carcinogen, 4-dimethylaminoazobenzene, produced parallel alterations in the carcinogenicity of this dye. In each series the 4'-isomer was the least active and the 3'-isomer was the most active. Furthermore, except for the very high activity of the 3'-methyl derivative, the activities of the various derivatives with substituents in any one position

TABLE I
ANALYSES ON THE PREVIOUSLY UNDESCRIBED AMINOAZO DYES

	M.P. °C. (corr.)	PER CENT N (DUMAS)* FOUND	THEORETICAL
Derivatives of 4-dimethylaminoazobenzene:			
2'-fluoro-	110.0-111.5	17.10, 17.68	17.27
3'-fluoro-	123.0-124.0	17.50, 17.66	"
4'-fluoro-	153.5-154.5	17.29, 17.41	"
2'-trifluoromethyl-	118.0-119.0	14.16, 14.36	14.33
3'-trifluoromethyl-	77.0-79.0	14.43, 14.77	"
4'-trifluoromethyl-	173.0-173.5	14.31, 14.33	"
2',5'-dichloro-	129.0-130.0	14.42, 14.57	14.29
2',4',6'-trichloro-	144.0-145.0	12.26, 12.49	12.79
2',3-Dimethyl-4-monoo-methylaminoazobenzene	83.5-84.5	17.84, 17.98	17.56
4-Formylmonomethylaminoazobenzene	110.5-111.5	17.94, 17.97	"

* These determinations were made by the Clark Microanalytical Laboratory, 104½ West Main Street, Urbana, Illinois.

were very similar. Apparently the position of the group in the molecule had a greater effect on the carcinogenicity of the dye than did the type of group involved.

An interpretation of these results as representing the effects *per se* of the nitro and methyl groups on the activities of these dyes *in vivo* must be made with caution since these groups may be converted *in vivo* to amino and carboxyl groups, respectively. Accordingly we are studying the effects of more stable groups on the carcinogenic-

ity of 4-dimethylaminoazobenzene. The present paper reports mainly on the activity of this dye after substitution in the 2', 3', or 4' positions with a) the *o*, *p*-directing fluoro, F-, group which forms the strongest known bond with carbon, and b) the highly stable *m*-directing and ring-deactivating trifluoromethyl, CF₃-, group. The results obtained with the former compounds bear on the problem of whether the occurrence of a benzidine rearrangement (4, 11) in the metabolism of aminoazo dyes could be of importance in the induction of tumors by these dyes. In addition the carcinogenic activities of several other dyes related to 4-dimethylaminoazobenzene are recorded.

METHODS

Preparation of compounds.—All but one of the compounds fed in these experiments were prepared in this laboratory. The melting points of the compounds which have been described previously agreed well with those in the chemical literature and are given in the text. Since several of the compounds have not been described previously, the nitrogen contents and corrected melting points of the new compounds are listed in Table 1. For these determinations 100 to 200 mg. of each compound was purified by treating a hot solution in benzene with charcoal, passing the cooled solution through 6" × 1" column of alumina, and finally crystallizing from benzene-petroleum ether (Skelly Solve B). The melting points of the compounds were raised no more than 1 or 2° C. by this purification.

Of the amines required for these syntheses aniline,¹ dimethylaniline,¹ *p*-fluoroaniline,² *m*-trifluoromethylaniline,² 2, 4, 6-tribromoaniline,¹ 2, 4, 6-trichloroaniline,^{1, 2} 5-dichloroaniline,¹ and *o*-toluidine¹ were obtained commercially. Dr. R. G. Jones of the Eli Lilly Research Laboratories very generously supplied us with the *o*- and *p*-trifluoromethylanilines; his syntheses of these difficultly obtainable amines have been published (10). The *o*- and

¹ Eastman Kodak Company, Rochester 4, N. Y.

² Fluor-Syn Laboratories, 1128 North Water St., Milwaukee 2, Wis.

* This investigation was supported in part by grants from the National Cancer Institute, U.S. Public Health Service and the Jane Coffin Childs Memorial Fund for Medical Research.

m-fluoroanilines were prepared by the general methods of Seliemani and Pillarsky (23), but the yields of the nitrobenzenediazonium borofluorates used in these preparations were increased by following the modifications introduced by Starkey (24). *m*-Hydroxydimethylaniline was prepared from *m*-nitroaniline¹ by methylation with dimethyl sulfate,¹ reduction of the nitro group with stannous chloride in concentrated HCl, and finally replacement of the amino group with a hydroxy group (1).

4-Dimethylaminoazobenzene (m.p. 117–118°), 2-hydroxy-4-dimethylaminoazobenzene (m.p. 163–165°), and the fluoro and trifluoromethyl derivatives of 4-dimethylaminoazobenzene were prepared by diazotizing 1 mole of the appropriate primary amine with 1 mole of sodium nitrite (25 per cent aqueous solution) in the presence of 2.5 moles of hydrochloric acid and about 30 moles of water at 0 to 3° C. For coupling, the diazo solution was added to a cooled (15° C.) 70 per cent ethanol solution of 1 mole of the desired tertiary amine in the presence of 2.5 moles of anhydrous sodium acetate. In the case of the fluoro and trifluoromethylanilines it was necessary to heat the acid mixture to dissolve the amine. The fine crystals of amine hydrochloride which appeared when the solution was cooled rapidly, went slowly into solution upon diazotization. The trifluoromethyl and 2-hydroxy derivatives of 4-dimethylaminoazobenzene were recrystallized from ethanol-water mixtures, while 4-dimethylaminoazobenzene and its fluoro derivatives were recrystallized from benzene-petroleum ether mixtures. The yields of the recrystallized dyes were approximately 80 per cent of theoretical.

2, 5-Dichloroaniline was diazotized by dissolving 0.1 mole in 50 ml. of 10 N hydrochloric acid and 210 ml. of water, cooling to 10° C., and then adding the sodium nitrite solution (0.1 moles) within 30 seconds (22). The diazo solution and dimethylaniline were then coupled as described above to give 2', 5'-dichloro-4-dimethylaminoazobenzene. After recrystallization from benzene and petroleum ether the yield was 68 per cent of theoretical.

Fifty per cent yields of 2', 4', 6'-tribromo-4-dimethylaminoazobenzene (m.p. 162–162.5° C.) and 2', 4', 6'-trichloro-4-dimethylaminoazobenzene were obtained by the general procedure outlined by Saunders (22). One-tenth mole of sodium nitrite dissolved in 70 ml. of concentrated sulfuric acid was added slowly to a solution of 0.1 mole of the halogenated amine in 40 ml. of concentrated sulfuric acid while the temperature was maintained below 20° C. The diazo solution was diluted by pouring it slowly into 2 to 3 times its volume of crushed ice and water and the mixture was added with efficient stirring to 350 ml. of cold 70 per cent ethanol containing 0.1 mole of dimethylaniline and 82 gm. of anhydrous sodium acetate. The dyes were recrystallized from a mixture of benzene and petroleum ether.

2', 3-Dimethyl-4-aminoazobenzene ("*o*-aminoazotoluene" or "*o*-amino-5-azotoluene") obtained commercially¹ was recrystallized from ethanol-water to a m.p. of 101 to 102° C. 2', 3-Dimethyl-4-monomethylaminoazobenzene was prepared from the diazoamino deriv-

ative formed by coupling diazotized *o*-toluidine with *N*-methyl-*o*-toluidine (19). The diazoamino compound was rearranged (19) by heating it at 47 to 50° C. for 70 minutes in the presence of excess *N*-methyl-*o*-toluidine and the hydrochloride of this amine. The dye was precipitated as the hydrochloride which was then decomposed with excess alkali. After recrystallization from benzene-petroleum ether the yield was 40 per cent of theoretical.

For the preparation of 4-formylmonomethylaminoazobenzene, 4-monomethylaminoazobenzene (16) was refluxed in toluene (15 ml. per gm. of dye) for $\frac{1}{2}$ hour with three equivalents of 88 per cent formic acid. The toluene was then distilled off slowly and, when less than half of the toluene remained, a second and identical charge of toluene and formic acid was added. After all of the toluene had been removed by distillation, the residue was taken up in ethanol, precipitated by the addition of water, and finally recrystallized from ethanol-water. 4-Monomethylaminoazobenzene was easily regenerated from the formyl derivative by acid hydrolysis. The preparations of 4'-nitro-4-dimethylaminoazobenzene (m.p. 227–229° C.) and 2, 4'-diamino-5-dimethylaminobiphenyl trihydrochloride have been described previously (19).

Method of assay.—Young adult male albino rats² weighing 160 to 190 gm. were housed in screen-bottom cages in groups of 5 to 7 with food and water available *ad libitum*. All of the compounds were fed in a semi-synthetic diet (13, diet 3) consisting of crude casein, 12; Vitab rice bran concentrate, 2; salts, 4; glucose monohydrate (earelose), 77; and corn oil, 5. After analysis of the dietary constituents sufficient crystalline riboflavin was added to bring the total content of the diet to the desired level. The rats of the first series (Table 2) received only 1 mg. of riboflavin per kg. of diet for the first 8 days. At that time the weight losses were so severe that the level was raised to 2 mg. per kg. of diet for the remainder of the first series and for the two subsequent experiments (Tables 3 and 4). In a fourth series the rats fed 4-dimethylaminoazobenzene and one of the groups fed the 4'-fluoro derivative received 2 mg. of riboflavin per kg. of diet while the diet for the second group fed 4'-fluoro-4-dimethylaminoazobenzene contained 10 mg. of riboflavin per kg. 4'-Nitro-4-dimethylaminoazobenzene and 2, 4'-diamino-5-dimethylaminobiphenyl trihydrochloride were added to the diets after being ground in a mortar with a small quantity of glucose; the other compounds were dissolved in the corn oil of the diet with mild heat. Each rat received 1 drop of halibut liver oil per month.

In the first series (Table 2) each compound was fed to 13 or 14 rats at a level of 2.67 millimoles per kg. of diet (equivalent to 0.060 per cent of 4-dimethylaminoazobenzene). At the end of 3 months the rats fed 4-dimethylaminoazobenzene and its 4'-fluoro derivative were examined for tumors by laparotomy. Those fed the 4'-fluoro derivative were then transferred to the basal diet (no dye added) for 2 months, while the other group was fed 4-dimethylaminoazobenzene for an additional

² Holtzman Rat Co., Madison, Wis.

month, examined by laparotomy a second time, and then fed the basal diet for 2 months. 2', 4', 6'-Tribromo-, 2', 5'-dichloro-, and 4'-nitro-4-dimethylaminoazobenzene were fed for 8 months before the rats were subjected to laparotomy and transferred to the basal diet for an additional 2 months. Since the rats would not survive with continuous feeding of 2', 4', 6'-trichloro-4-dimethylaminoazobenzene, they were transferred to the basal diet for 1 month periods at the end of the fifth and seventh months of dye-feeding. The total period during which the dye was actually ingested was 10 months. 2', 3-Dimethyl-4-aminoazobenzene and its N-monomethyl derivative were fed continuously for 14 months.⁴

In the second series (Table 3) 2.40 millimoles of 4-dimethylaminoazobenzene and its three fluoro derivatives were fed per kg. of diet for 3 months while 2-hydroxy-4-dimethylaminoazobenzene was fed at a level of 2.67 millimoles per kg. for 8 months. At the end of the dye-feeding periods the livers were examined by laparotomy, and after 2 months on the basal diet the rats were killed for a final tumor count.

In the third series (Table 4) 4-dimethylaminoazobenzene and its three trifluoromethyl derivatives were at first fed at a level of 2.67 millimoles per kg. Due to the poor health of the rats fed the 2'- and 3'-trifluoromethyl-derivatives it was necessary after 5 weeks to transfer the rats fed 4-dimethylaminoazobenzene and the trifluoromethyl derivatives to the basal diet for 10 days, and then feed a reduced level of these four dyes (2.13 millimoles per kg.) for the remainder of the experiment. After 4-dimethylaminoazobenzene had been fed for a total of 5 months and the trifluoromethyl derivatives and 4-formylmonomethylaminoazobenzene (2.67 millimoles per kg.) for 8 months, the livers were examined by laparotomy and the animals maintained on the basal diet for 2 months. 2, 4'-Diamino-5-dimethylaminobiphenyl trihydrochloride was fed at the higher level of 5.34 millimoles per kg. for 10 months. Each group in series two and three consisted of 14 rats.

In the fourth series both 4-dimethylaminoazobenzene and its 4'-fluoro derivative were fed at a level of 2.40 millimoles per kg. of diet. Three rats from each of the groups fed 4-dimethylaminoazobenzene and 4'-fluoro-4-dimethylaminoazobenzene in the low riboflavin (2 mg. per kg.) diet were killed for hepatic riboflavin (2) and protein-bound dye (12, 15) analyses at 1, 2, 3, 4, 6, 8, 13, and 18 weeks. These analyses were also made on the livers of 3 rats fed 4'-fluoro-4-dimethylaminoazobenzene in the high riboflavin (10 mg. per kg.) diet and the basal diet containing low or high levels of riboflavin for 3, 8, and 18 weeks. To determine the effect of the level of dietary riboflavin on the carcinogeneity of 4'-fluoro-4-dimethylaminoazobenzene the livers of 17 and 13 rats fed the dye in the diets containing the low and high levels of riboflavin, respectively, were examined by laparotomy at 18 weeks. They were then fed basal diets

containing the same levels of riboflavin for an additional 2 months before being killed for the final tumor count.

After the dyes which had not previously been tested for carcinogenic activity were fed for at least 2 weeks in the above experiments the absorption of the compounds from the gastrointestinal tract was investigated by determining the dye content of 24 hour fecal samples. The feces were air-dried at 37° C., powdered, and extracted with acetone in a Soxhlet apparatus for at least 24 hours. An aliquot of the acetone extract was evaporated to dryness *in vacuo*, the residue was dissolved in 7 N HCl, and the light absorption of this solution was compared with that of a standard solution of the compound fed.

RESULTS

The results of the three series in which a total of 14 aminoazo dyes and one possible non-azo metabolite of 4-dimethylaminoazobenzene were tested for carcinogenicity are tabulated in Tables 2 to 4. The tumor incidences of the three control groups fed 4-dimethylaminoazobenzene agreed well with those obtained previously (19, 21). The other compounds had activities ranging from zero to nearly twice that of the reference compound.

Although the carcinogenicity of 2', 3-dimethyl-4-aminoazobenzene ("o-aminoazotoluene") for the rat liver has been definitely established (8), no tumors⁴ were obtained in this experiment when it was fed at a level of 2.67 millimoles per kg. of diet for 14 months (Table 2, group 7). Likewise, no liver tumors⁴ were found when its N-methyl derivative, 2', 3-dimethyl-4-monomethylaminoazobenzene (group 8) was fed for 14 months at the same level. However, two of the rats fed the latter compound had tumors at other sites; one developed a carcinoma arising from the sebaceous glands near the eye after 12 months while a mixed basal and squamous cell carcinoma arising from the ear duct was found in a second rat after 14 months.⁵ This ear tumor was similar to those found after administration of certain amines such as 2-acetylaminofluorene (26), certain heterocyclic analogues of the latter compound (14), and the 4-aminostilbenes (7).

The polyhalogen derivatives 2', 4', 6'-tribromo-, 2', 4', 6'-trichloro-, and 2', 5'-dichloro-4-dimethylaminoazobenzene (Table 2, groups 3-5) were all inactive even after being fed for 8 to 10 months, although the trichloro derivative did produce mild cirrhosis and cysts in the liver after being

⁴ Note added in proof: After these rats had been fed the dye for a total of 16 months they were continued on the basal diet for 2 more months and then killed. By this time 2', 3-dimethyl-4-aminoazobenzene had produced benign hepatomas in 2 of the remaining 9 rats; 2 of the 10 rats that had been fed the N-methyl derivative developed similar tumors.

⁵ We are indebted to Drs. H. P. Ruseh and J. M. Price for the histological examinations of the tumors reported in this paper.

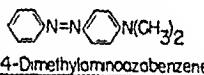
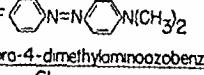
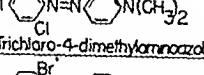
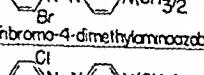
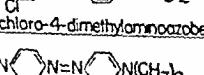
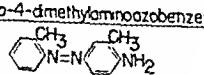
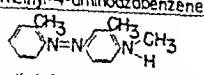
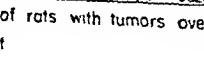
fed for 8 to 10 months. 4'-Nitro-4-dimethylaminoazobenzene (group 6) was likewise non-carcinogenic in these tests; this compound was fed to complete the series of derivatives containing a nitro group on the prime ring (19). The inactivity of the tribromo and 4'-nitro derivatives is of doubtful significance, however; in each case approximately half of the dye fed was apparently not absorbed since it was excreted in the feces unchanged. On the other hand no more than 3 per cent of the 2', 4', 6'-trichloro-4-dimethylaminoazobenzene and less than 1 per cent of the other compounds reported in this paper were excreted in the feces.

In the first series 4'-fluoro-4-dimethylaminoazobenzene (group 2) induced liver tumors in 7 of 12 rats by 3 months while none of the rats fed 4-dimethylaminoazobenzene had gross tumors at that time. Since all other substitutions in the 4' position have resulted in compounds considerably less active than 4-dimethylaminoazobenzene (17, 19), it was of interest to feed the 2'- and 3'-fluoro derivatives as well. In this series the 4'- and 3'-

fluoro derivatives (Table 3, groups 10 and 11) appeared to be equally active and almost twice as carcinogenic as 4-dimethylaminoazobenzene. 2'-Fluoro-4-dimethylaminoazobenzene (group 12) induced tumors a little faster than 4-dimethylaminoazobenzene in this experiment, but the difference was small. In the same experiment 2-hydroxy-4-dimethylaminoazobenzene (group 13), a possible metabolite of the parent compound, did not produce any tumors or gross liver damage after being fed for 8 months; the 2', 3', and 4'-hydroxy derivatives are also non-carcinogenic (19).

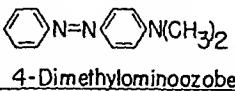
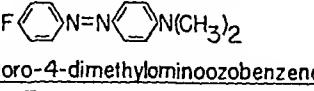
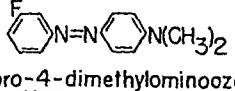
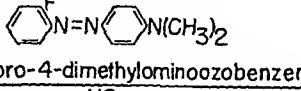
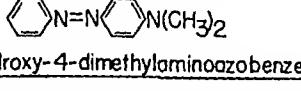
While replacement of a hydrogen on the benzene ring with a fluorine atom enhanced the activity of 4-dimethylaminoazobenzene, replacement of the three hydrogens of the methyl groups of 3'- and 2'-methyl-4-dimethylaminoazobenzene by fluorine destroyed their activities (Table 4, groups 16 and 17; cf. Table 5). 4'-Trifluoromethyl-4-dimethylaminoazobenzene (group 15) was likewise inactive when fed for 8 months. The rats receiving the 2'- and 3'-trifluoromethyl derivatives were in

TABLE 2
THE CARCINOGENICITIES OF CERTAIN HALOGEN DERIVATIVES
OF 4-DIMETHYLAMINOAZOBENZENE

Group	Compound fed (2.67 millimoles per kgm. diet)	Time dye was fed mos.	Incidence of liver tumors*							Gross cirrhosis of end of feeding dye
			3	4	6	8	10	12	14	
1		4	0/13	3/13	10/13					mild-moderate
2		3	7/12	12/12						moderate-severe
3		10 [†]					0/9	0/9		none-mild
4		8				9/11	9/11			none
5		"				0/10	0/10			"
6		"				0/12	0/12			"
7		14						0/9		"
8		"						0/10		"

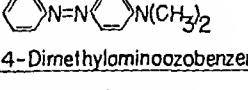
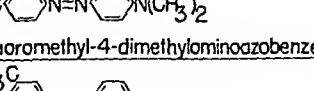
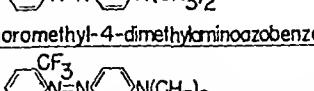
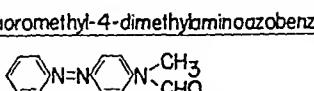
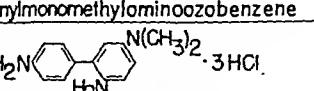
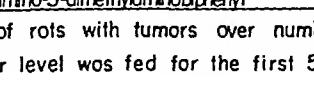
* Number of rats with tumors over number of rats alive at end of feeding dye
[†] See text

TABLE 3
THE CARCINOGENICITIES OF CERTAIN MONOFLUORO DERIVATIVES OF 4-DIMETHYLAMINOAZOBENZENE

Group	Compound fed	Level in diet millimoles per kgm.	Time dye was fed mos.	Incidence of liver tumors* months				Gross cirrhosis at end of feeding dye
				3	5	8	10	
9		2.40	3	1/14	8/14			mild
10		"	"	8/13	12/13			mild-severe
11		"	"	8/13	12/13			moderate-severe
12		"	"	4/14	9/14			mild-moderate
13		2.67	8			0/14	0/14	none

*Number of rats with tumors over number of rats alive at end of feeding dye

TABLE 4
THE CARCINOGENICITIES OF CERTAIN TRIFLUOROMETHYL DERIVATIVES OF 4-DIMETHYLAMINOAZOBENZENE

Group	Compound fed	Level in diet millimoles per kgm.	Time fed mos.	Incidence of liver tumors* months					Gross cirrhosis at end of feeding compound
				5	7	8	10	12	
14		2.67 [†] 2.13	5	6/13	9/13				mild-moderate
15		"	8			0/11	0/11		none
16		"	"			0/8	0/8		"
17		"	"			0/9	0/9		"
18		2.67	"			2/11	4/11		none-mild
19		5.34	10				0/11	0/11	none

* Number of rats with tumors over number of rats alive at end of feeding compound

† The higher level was fed for the first 5 weeks and the lower level for the remainder of the experiment

poor health during the first month of the experiment so that it was necessary to feed the basal diet for 10 days and then continue with a reduced level of dye. 4-Formylmonomethylaminoazobenzene (group 18), a possible intermediate in the formation of 4-monomethylaminoazobenzene from the dimethyl compound *in vitro*, was much less active than either of these compounds. Although it was fed for 8 months, the compound induced liver tumors in only 4 of 11 rats by 10 months. 2, 4'-Diamino-5-dimethylaminobiphenyl trihydrochloride (group 19), which has been suggested as a possible metabolite of 4-dimethylaminoazobenzene (3, 4), did not induce any liver tumors even though it was fed at a level of 5.34 millimoles per kg. of diet for 10 months. This confirms an earlier test in which the compound was fed for only 4 months (19). An adenocarcinoma arising from the small intestine was found in one of the surviving rats which were killed at 12 months.

The liver tumors induced by the three fluoro dyes and 4-formylmonomethylaminoazobenzene were histologically similar to those induced by 4-dimethylaminoazobenzene.⁵

Studies on 4'-fluoro-4-dimethylaminoazobenzene.—Like 4-dimethylaminoazobenzene (21) the carcinogenicity of 4'-fluoro-4-dimethylaminoazobenzene was markedly altered by the riboflavin level of the diet. Thus when the 4'-fluoro derivative was fed in a diet containing 2 mg. of riboflavin per kg. for 3.5 months, the tumor incidence was 41 per cent at 3.5 months and 82 per cent after 2 months on the basal diet. The corresponding incidences for the rats fed 10 mg. of riboflavin per kg. of diet were 0 and 15 per cent respectively. Further, the livers of the rats fed the high level of riboflavin showed little or no gross damage while the livers of those fed the lower level were moderately cirrhotic. In this experiment only one of 11 rats fed 4-dimethylaminoazobenzene for 3.5 months had gross tumors at that time.

The ingestion of either 4-dimethylaminoazobenzene or its 4'-fluoro derivative in the low riboflavin diet decreased the level of hepatic riboflavin from 14.4 μgm . per gm. at the beginning of the experiment to approximately 11.6 and 10.6 μgm . per gm. by 3 and 8 weeks respectively. The livers of rats fed the same diet without the dye contained 12.8 and 17.3 μgm . of riboflavin per gm. at 3 and 8 weeks respectively. Ingestion of the high riboflavin diet increased the level of hepatic riboflavin to 17.5 and 18.1 μgm . per gm. for the rats fed the basal diet and 14.3 and 15.4 μgm . per gm. for those fed 4'-fluoro-4-dimethylaminoazobenzene at 3 and 8 weeks respectively. High levels of dietary riboflavin also increase the level

of hepatic riboflavin which can be maintained when either 4-dimethylaminoazobenzene (13) or its 3'-methyl derivative is fed (6).

Like the other dimethylaminoazo dyes which have been studied (15) 4'-fluoro-4-dimethylaminoazobenzene gave rise to a considerable level of protein-bound aminoazo dye in the liver. In this experiment the level of bound dye reached a maximum between 3.5 and 4 weeks when either 4-dimethylaminoazobenzene or its 4'-fluoro derivative was fed in the low riboflavin diet (Fig. 1). After the maximum levels were reached, the level of bound dye derived from the 4'-fluoro derivative

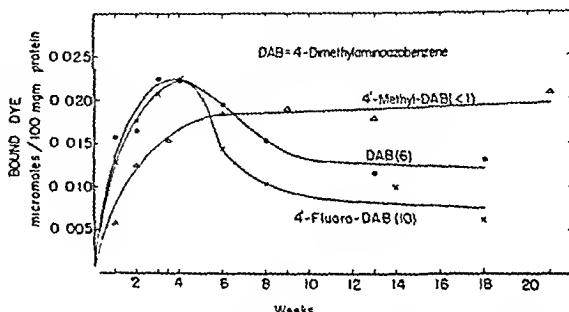


FIG. 1.—The protein-bound dye-time curves for 4-dimethylaminoazobenzene and its 4'-fluoro and 4'-methyl (15) derivatives. The relative carcinogenic activities of the dyes are given in parentheses. The micromolar level of the bound dye derived from 4'-fluoro-4-dimethylaminoazobenzene was calculated from the quantity of steam-distillable amine obtained following reduction with tin and hydrochloric acid according to the general procedure described previously (15). One micromole of amine was obtained from a solution of the bound dye which had a light absorption ($\log I_0/I$) at 520 μm of 12.0 when dissolved in 4.5 ml. of the hydrochloric acid-ethanol solution. The constant for 4-dimethylaminoazobenzene is also 12.0 under these conditions (15).

fell more rapidly than that formed from 4-dimethylaminoazobenzene; a similar sharp drop in the level of bound dye after the maximum was found when the very active 3'-methyl-4-dimethylaminoazobenzene was fed (15). The levels of bound dye in the livers of the rats fed 4'-fluoro-4-dimethylaminoazobenzene in the high riboflavin diet for 3, 8, or 18 weeks were similar to those observed when the low riboflavin diet was fed. The period of dye-feeding required before the maximum level of protein-bound dye derived from the 4'-fluoro derivative was obtained fits in reasonably well with similar data on the C-monomethyl derivatives of 4-dimethylaminoazobenzene (15). Thus the bound dye-time curve for the very active 3'-methyl derivative (activity = 10–12) reached a maximum after 2 weeks of dye-feeding while the bound dye-time curves with 4-dimethylaminoazobenzene (activity = 6), 2'-methyl-4-dimethylaminoazoben-

zene (activity = 2-3), and 4'-methyl-4-dimethylaminoazobenzene (activity <1) reached maxima after approximately 4, 8, and <21 weeks, respectively. 2-Methyl-4-dimethylaminooazobenzene (activity = 0) and 3-methyl-4-monomethylaminoazobenzene (activity <1) gave rise to maximum bound dye levels after 12 weeks of feeding.

DISCUSSION

The data presented above on the activities of the halogen-containing derivatives of 4-dimethylaminoazobenzene have a bearing on the benzidine-rearrangement hypothesis of Elson and Warren (4). These workers found that the urine from rats fed azobenzene, a non-carcinogenic compound, contained a substance which yielded benzidine or 4, 4'-diaminobiphenyl on treatment

TABLE 5
THE CARCINOGENICITIES OF VARIOUS RING-SUBSTITUTED DERIVATIVES OF 4-DIMETHYLAMINOAZOBENZENE

position	Relative Activities (unsubstituted dye = 61)						
	HO-	CH ₃ -	NO ₂ -	Cl-	F-	CF ₃ -	
4'	0	<1	0	1-2	10	0	
3'	0	10-12	5	5-6	10	0	
2	0	2-3	3	2	7	0	
2	0	0					
3	0						

with acid. This substance was assumed to be a derivative of hydrazobenzene and it was suggested that a rearrangement of this compound to benzidine might also take place *in vivo*. Elson and Hoch-Ligeti (3) also considered it probable that 4-dimethylaminoazobenzene undergoes a similar rearrangement *in vivo* to yield 2, 4'-diamino-5-dimethylaminobiphenyl. This compound, a substituted *p*-phenylene diamine, was found to be a potent enzyme inhibitor (3) and hence might be involved in the carcinogenic process induced by the parent dye. Since at least one of the *para* or *ortho* positions of aromatic hydrazo compounds must be unsubstituted for the benzidine rearrangement to take place (9), the substitution of one or more groups in the 2', 4', and 6' positions of 4-dimethylaminoazobenzene should prevent, or at least hinder, this reaction from occurring *in vivo*. Actually neither 2', 4', 6'-trichloro- or 2', 4', 6'-tribromo-4-dimethylaminoazobenzene was active. While the inactivity of the tribromo com-

pound can be disregarded since no more than half of the compound fed was absorbed, the inactivity of the triehloro derivative is negative evidence in support of the benzidine hypothesis. On the other hand, the very high activities of the 4'- and 2'-fluoro derivatives constitute positive evidence against the hypothesis for the carbon-fluorine bonds in these compounds are among the strongest known chemical linkages. Since *para* rearrangements usually take place with more facility than those directed to the *ortho* positions, it might be expected according to this theory that the 4'-fluoro derivative would have a very low activity; instead it proved to be *more* active than the parent dye or the 2' derivative. Furthermore, the direct tests of the rearrangement product itself for carcinogenic activity in the rat liver have so far yielded negative results (Table 4, group 19 and (19)). In any case the fact that substitution of the fluorine atom into any of the positions on the prime ring of 4-dimethylaminoazobenzene did not decrease the activity of this dye encourages one to attempt further tests of the benzidine rearrangement hypothesis by preparing polyfluoro derivatives substituted in such a way that they could not undergo this rearrangement upon reduction to the corresponding hydrazo compounds.

The data presented in this and previous work (17, 19) concerning the effect of substitution in the rings of 4-dimethylaminoazobenzene on its carcinogenicity are summarized in Table 5. It is evident that chloro, nitro, and methyl groups act alike in producing similar series of compounds of graded carcinogenicities. Hydroxy and trifluoromethyl groups are also alike in that they destroy the ability of the molecule to act as a carcinogen. The effect of the hydroxy group may be to provide a point of attack for metabolic breakdown of the dye before it can act carcinogenically. In fact, 4'-hydroxy-4-dimethylaminoazobenzene is formed from 4-dimethylaminoazobenzene during its metabolism by rat liver homogenates (20) and there is evidence that demethylated hydroxyazo derivatives are present in the urine of rats fed the dye (18). It is not known how the strongly *m*-directing and ring-deactivating trifluoromethyl group destroys the carcinogenic activity of the dye. Equally difficult to explain are the observations that the fluoro group in the 3' and 4' positions increases the activity of the molecule while the same group in the 2'-position did not alter the activity of the dye. This is particularly striking in the ease of the 4'-derivative since other substituents in this position either abolish or greatly diminish the activity of 4-dimethylaminoazobenzene. It is interesting that the activity of the 4'-fluoro derivative,

like that of the parent dye, can be strongly inhibited by feeding a high level of riboflavin. On the other hand, the activity of 3'-methyl-4-dimethylaminoazobenzene, another derivative that is more active than the parent dye, is not greatly reduced by dietary additions of this vitamin (5). However, like the other highly active aminoazo dyes (15) the amount of protein-bound dye formed from 4'-fluoro-4-dimethylaminoazobenzene rises to a maximum within 1 month of dye-feeding and then diminishes. This is quite unlike the bound dye-time curve of 4'-methyl-4-dimethylaminoazobenzene, a very weak carcinogen ((15) and Fig. 1).

Although it was expected from the data in the literature (8) that 2', 3-dimethyl-4-aminoazobenzene ("o-aminooazotoluene") would be a very weak carcinogen under our conditions, the substitution of a *N*-methyl group did not increase its activity for rat liver to a measurable extent. While this result might not have been expected in view of the great increase in activity effected by the monomethylation of 4-aminoazobenzene (17, 19, 25) it was known that a methyl group in the 3 position does diminish strongly the activity of 4-monomethylaminoazobenzene (19). Similarly, the low activity of 4-formylmonomethylaminoazobenzene, a possible metabolite of 4-dimethylaminoazobenzene, was surprising. Evidently the *N*-formyl group in this compound is not readily removed *in vivo* to yield the highly active dye, 4-monomethylaminoazobenzene.

While it is difficult to evaluate the importance of steric hindrance in determining the carcinogenic activities of the compounds listed in Table 5, this factor should probably receive more attention. The "space-filling" properties of the groups under discussion appear in many cases to be of as much importance as their effects on the electronic structure of the whole molecule. These considerations may apply, for example, to such inactive dyes as 2', 4', 6'-trichloro-4-dimethylaminoazobenzene and the trifluoromethyl derivatives of 4-dimethylaminoazobenzene.

SUMMARY

- The carcinogenic activities of 15 halogen and other derivatives of the hepatic carcinogen, 4-dimethylaminoazobenzene, were determined in the rat. Some of these compounds were fed to test the hypothesis (Elson and associates) that the parent dye undergoes a benzidine rearrangement *in vivo* which is concerned in the carcinogenic process induced by the dye.

- The substitution of the stable *o*, *p*-directing fluoro group in the 3' or 4' positions yielded dyes which were nearly twice as active as 4-dimethyl-

aminoazobenzene, whereas the 2'-fluoro derivative had essentially the same activity as the parent dye. The activity of the 4'-fluoro derivative was strongly inhibited by high levels of dietary riboflavin, and the level of protein-bound dye formed in the liver from this dye reached a maximum after 1 month of feeding.

- The substitution of the stable *m*-directing trifluoromethyl group in the 2', 3', or 4' positions yielded dyes that were inactive even after 8 months of continued administration.

- Two dyes that presumably could not undergo the benzidine rearrangement *in vivo*, 2', 4', 6'-tribromo- and 2', 4', 6'-trichloro-4-dimethylaminoazobenzene, were inactive, but the tribromo derivative was only poorly absorbed from the intestinal tract. However, the benzidine rearrangement product of 4-dimethylaminoazobenzene, 2, 4'-diamino-5-dimethylaminobiphenyl, was inactive even when fed at a high level for 10 months. In addition, the high activities of the 2'- and 4'-fluoro derivatives are considered to constitute positive evidence against the benzidine rearrangement hypothesis.

- Two possible metabolites of 4-dimethylaminoazobenzene were tested: 4-formylmonomethylaminoazobenzene had only a low carcinogenic activity and 2-hydroxy-4-dimethylaminoazobenzene was inactive. The following miscellaneous derivatives were inactive under the conditions used: 2', 3-dimethyl-4-aminoazobenzene ("o-aminooazotoluene"), 2', 3-dimethyl-4-monomethylaminoazobenzene, and 4'-nitro-4-dimethylaminoazobenzene.

- The following new compounds are described: 2'-fluoro-, 3'-fluoro-, 4'-fluoro-, 2'-trifluoromethyl-, 3'-trifluoromethyl-, 4'-trifluoromethyl-, 2', 4', 6'-trichloro-, and 2', 5'-dichloro-4-dimethylaminoazobenzene; 2', 3-dimethyl-4-monomethylaminoazobenzene and 4-formylmonomethylaminoazobenzene.

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Electrophoretic, Nitrogen, Lipide and Enzyme Studies of the Plasma and Plasma Fractions in Cancer*

GEORGE H. L. DILLARD, H. ROWLAND PEARSALL, AND ALFRED CHANUTIN

(From the Biochemical Laboratory, Medical School, University of Virginia, Charlottesville, Virginia)

Electrophoretic and chemical studies of the plasma and plasma fractions of injured animals and diseased humans show marked variations from the respective controls (5, 6, 7, 13). The available information concerning the detailed changes of plasma proteins of patients with cancer is limited. This paper is concerned with the electrophoresis and chemistry of plasma and three plasma protein fractions together with data for three typical plasma enzymes in a group of patients with various types of cancer.

METHODS

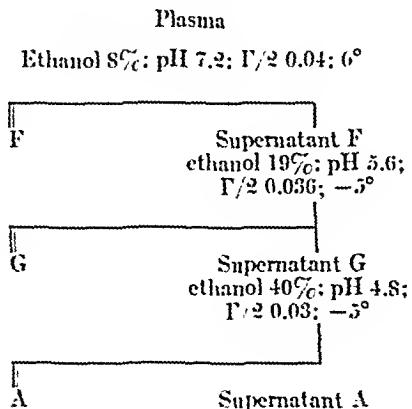
The diagnosis for each patient is established. The diagnoses for the 36 cases studied are as follows: carcinoma of the bronchus, 3; pancreas, 3; prostate, 9; stomach, 5; colon, 2; breast, 3; ovary, 1; kidney, 1; larynx, 1; hepatoma, 1; myelogenous leukemia, 2; and lymphoblastoma, 5. These cases are free of detectable intercurrent infection or massive necrosis of tumor tissue. The control group consists of 17 healthy young men.

Fasting samples of blood (50 ml.) are drawn into heparin-moistened syringes and are centrifuged immediately in Lastroid tubes. The fractionation of the separated plasma is begun within 30 to 60 minutes after the blood is collected.

A relatively simple procedure for fractionating plasma is outlined in Diagram 1.

DIAGRAM 1

FRACTIONATION OF PLASMA¹



1. The plasma (18 ml.) is adjusted to pH 7.2 with 3 to 4 ml. of phosphate buffer (pH 6.4, $\Gamma/2$ 0.15). Calculated amounts of water are added, the mixture cooled to 0 to 1.0°, and the ethanol concentration adjusted to 8 per cent with 95 per cent ethanol. The 5 \times diluted plasma is stirred for 2 minutes and fraction F is removed by centrifuging at 0°.

2. Supernatant F is adjusted to pH 5.6 with an acetate-acetic acid buffer (pH 4.0; $\Gamma/2$ 0.8) and sufficient 95 per cent ethanol is added to give a final concentration of 19 per cent. The final volume is 5.5 \times the original plasma volume. Fraction G is removed by centrifuging at -5°.

3. Supernatant G is adjusted to pH 4.8 with about 0.8 ml. of acetate-acetic acid buffer (pH 4.0, $\Gamma/2$ 0.4) and sufficient 95 per cent ethanol is added to yield a final concentration of 40 per cent. This represents a 7 \times dilution.

The three fractions are dissolved in 0.85 per cent saline and brought to 10 ml. volumes shortly after precipitation.

Aliquots of each fraction are diluted with equal amounts of barbiturate-NaOH buffer (pH 8.6, $\Gamma/2$ 0.1) and dialyzed against 2 liters of this buffer for 3 days in the cold. Electrophoresis is carried out in the Tisclius apparatus according to Longsworth's modification of the schlieren method (11) in a micro cell of 2 ml. capacity.² The ascending patterns are used for analysis and no attempt is made to calculate mobilities.

The plasma and fractions are analyzed for lipide carbon (18), cholesterol (16), and nitrogen.

* This investigation was supported by research grants from the National Cancer Institute, U.S. Public Health Service, and from the Office of Naval Research.

¹ The first step is similar to the procedure for obtaining Fraction I in Method 6 of Cohn et al. (3). Most of the fibrinogen is thus removed and avoids the clotting that is occasionally observed. The second step depends on the observation made by Cohn and associates (4) that practically all the globulins are precipitated by adjusting diluted plasma to pH 5.6 and 19 per cent ethanol at -5°. The conditions for obtaining the last fraction are similar to those used in Method 6 (3) for obtaining Fraction V. The nomenclature of plasma protein fractions is still confused and must await clarification by such workers as those at Harvard who are intensively studying this problem. Consequently, the 3 fractions obtained in this work are named F, G and A as a matter of convenience to indicate that fibrinogen, globulin and albumin are present in appreciable amounts in each of the respective fractions.

² Purchased from Pyrocell Mfg. Co., New York City.

Alkaline and acid phosphatases are estimated by the methods of Binkley, Shank, and Hoagland (2). Amylase is determined according to the procedure recommended by Andersch (1). The activity of these enzymes decreases particularly in the fractions, when stored at temperatures slightly above freezing. Quick-freezing of the samples prevents this deterioration and therefore all analyses are done immediately, or after storage in the frozen state. The nitrogen, lipide carbon, cholesterol content, and enzyme activities are expressed on the basis of 100 ml. of whole plasma.

ELECTROPHORETIC ANALYSES OF WHOLE PLASMA AND FRACTIONS

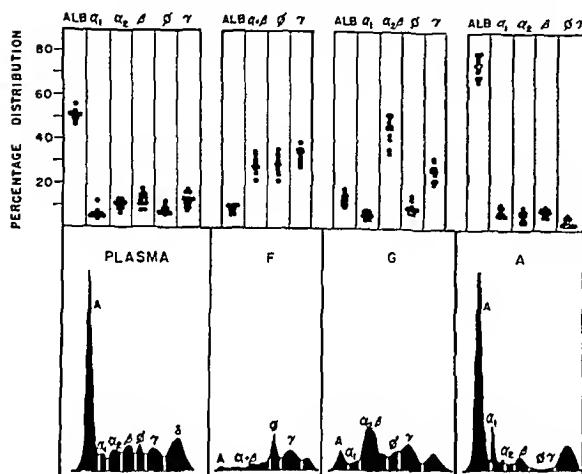


FIG. 1.—The distribution of the protein components of plasma and plasma fractions of healthy young men.

RESULTS

Typical electrophoretic patterns and the percentage distributions of the protein components of the plasma and 3 plasma fractions of healthy young men are shown in Figure 1. Fraction F contains most of the fibrinogen and small amounts

of α_1 , β and γ globulins. According to Morrison (12), most of these globulins are occluded by fibrinogen under the precipitation conditions. The greater portion of the β and γ globulins of the plasma are present in fraction G. The last fraction probably contains α_1 lipoprotein, α_2 glycoprotein, the β_1 metal combining protein as well as most of the plasma albumin.

The electrophoretic percentage distributions of the protein components of the plasma and plasma fractions of cancer patients are shown graphically in Figure 2. The most striking changes noted are the consistently low values for albumin in the plasma and in fraction A and the increases in the fibrinogen of fraction F.

About 75 per cent of the plasma nitrogen values of cancer patients are within the control range (Fig. 3). No correlation between the degree of cachexia and physical findings and the nitrogen concentration of the plasma is possible. About one-third of the values for fraction F are elevated. No consistently abnormal distribution for the fraction G values is seen. Approximately 80 per cent of the fraction A values are below the control range.

The distributions of lipide carbon and cholesterol in plasma and in fraction G are shown in Figure 4. Fractions F and A contain small amounts of cholesterol. No explanation can be offered for the lipide carbon values of the plasma which fall outside the control range. Most of the cholesterol values of the plasma and fraction G are in the lower portion or below the control range. The values for the per cent free cholesterol of the plasma are elevated in about 40 per cent of the patients but are not as marked in fraction G. The two highest values of 53 and 75 per cent are obtained in two cases of carcinoma of the pancreas with common duct obstruction. The remaining

ELECTROPHORETIC ANALYSES OF WHOLE PLASMA AND FRACTIONS

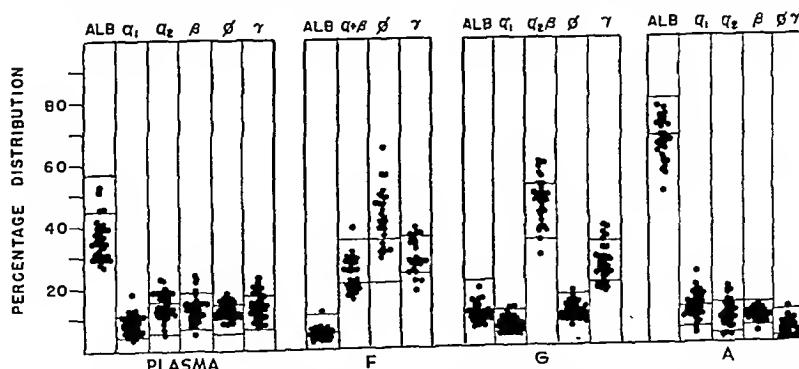


FIG. 2.—The distribution of the protein components of plasma and plasma fractions of cancer patients. The rectangles represent the respective control ranges.

elevated values cannot be related to any signs of liver damage.

Data for alkaline and acid phosphatase and for amylase in plasma and plasma fractions are shown in Figure 5. The alkaline phosphatase of the plasma and fraction A is elevated in about 50 per cent of the cases; a greater proportion of the fraction G values are above the control level. It is worthwhile pointing out that the activity of the alkaline phosphatase of fraction A is consistently greater than that of fraction G in each case.

The highest values for acid phosphatase are encountered in the plasmas of 5 patients with disseminated carcinoma of the prostate. When the plasma values are elevated, the enzyme is generally increased to about the same extent in fractions F and A.

Large variations from the control range are observed in the amylase content of the plasma, and fractions G and A. Attempts to account for these changes on the basis of clinical and pathologic observations are not successful.

DISCUSSION

It is generally concluded from electrophoretic studies of the plasmas of cancer patients that the changes observed are usually nonspecific (9, 10, 14, 15, 17). Pearsall and Chanutin (13) conclude that the degree of alteration in the plasma proteins appears to be determined by the severity of the disease. Generally, tissue destruction, infection, and cachexia are accompanied by a decrease in plasma albumin and usually by an increase in

fibrinogen and possibly other globulins. The electrophoretic, nitrogen and lipide analyses of plasma fractions in these experiments do not aid in distinguishing cancer from other diseases.

Greenstein (8) has pointed out that alkaline phosphatase is elevated in metastases to bone and in lymphoid disease. In the present study increases in the activity of this enzyme are observed in all

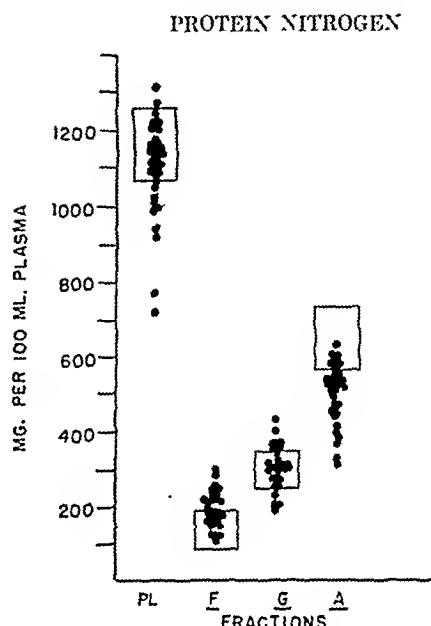


FIG. 3.—The distribution of protein nitrogen in the plasma and three plasma fractions of cancer patients. The rectangles represent the control ranges.

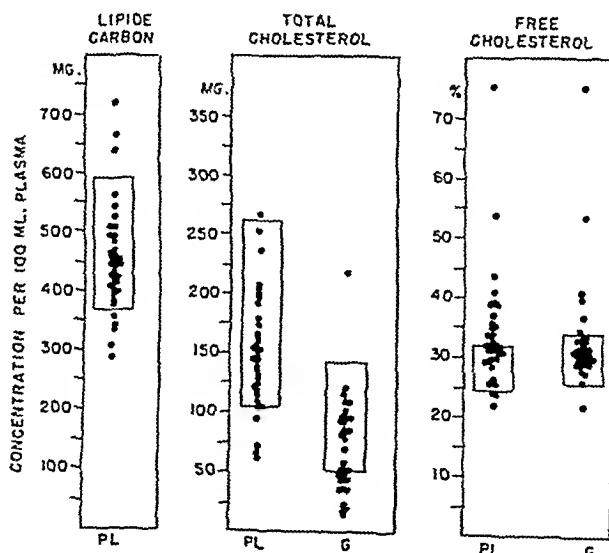


FIG. 4.—The distribution of lipide carbon in plasma and cholesterol in plasma and Fraction "Globulin" in cancer patients. The rectangles represent the control ranges.

cases with bone metastases and in 4 of the 7 cases with lymphoid malignancies. The marked changes in acid phosphatase are limited to disseminated carcinoma of the prostate. The amylase activity of the plasma and its fractions is too varied for interpretation. It appears that cachexia is not necessarily a factor in determining the changes in amylase concentration.

SUMMARY

Methods are outlined for fractionating small volumes of plasma into fibrinogen, globulin, and albumin-rich fractions by the low temperature-ethanol procedures.

Electrophoretic, nitrogen, lipide carbon, cholesterol and enzymic analyses of the plasma and three plasma fractions of 17 healthy young men and of

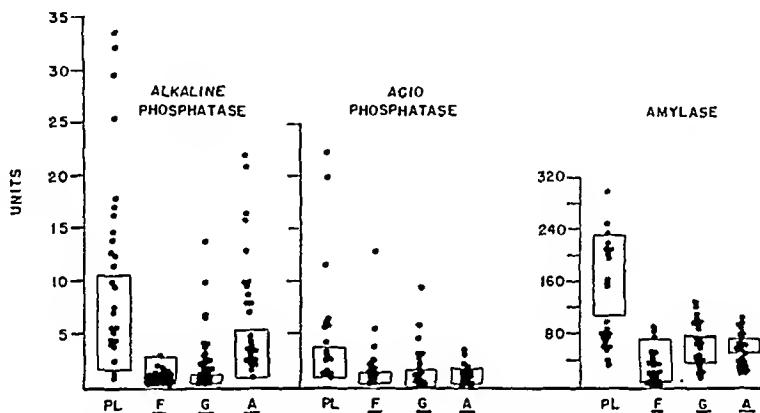


FIG. 5.—The distribution of three enzymes of plasma and three plasma fractions of cancer patients. The rectangles represent the control ranges.

36 patients with carcinoma of the prostate, stomach, pancreas, bronchus, breast, colon, ovary, and larynx; lymphoblastoma, myelogenous leukemia, and hepatoma are presented. The electrophoretic and nitrogen analyses show: (a) a consistent decrease in albumin, (b) an increase in fibrinogen, and (c) few significant changes in the globulin components. The changes in the lipides of plasma and its fractions are due to cachexia or hepatic involvement. The distributions of the alkaline and acid phosphatases and amylase of plasma fractions are discussed.

The results presented for the plasma protein fractions do not yield information which is of value in the diagnosis of cancer.

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The Protease and Antiprotease of Plasmas of Patients with Cancer and Other Diseases*

GEORGE H. L. DILLARD AND ALFRED CHANUTIN

(From the Biochemical Laboratory, Medical School, University of Virginia, Charlottesville, Va.)

MacFarlane and Biggs (5) recently reviewed the literature dealing with the plasminogen-plasmin, an active proteolytic enzyme and an antiprotease which are present in plasmas in health and disease. Satisfactory quantitative data for the plasminogen-plasmin relationship and for the spontaneously active proteolytic enzyme in disease are limited. Although the antiprotease has been studied rather extensively, its relationship to the proteolytic enzymes is not well established.

The active proteolytic enzyme observed in the plasmas of diseased individuals has been designated chiefly as fibrinolysin and "plasmin." The characterization of this enzyme is inadequately defined. With a few exceptions (5), the results obtained for the activities of this proteolytic enzyme in the plasmas of patients cannot be considered as quantitative.

Von Dinger (2) first attempted to determine the trypsin inhibitor quantitatively in the plasmas of patients with osteomyelitis. Subsequently, many observations have been made on antiprotease with a variety of procedures. It is generally agreed that this inhibitor is increased in a large variety of diseases (3, 4). Christensen (1) studied the kinetics of the inhibitor-crystalline trypsin reaction and was able to develop a well standardized procedure for determining inhibitor activity.

This investigation is concerned with the plasmin, spontaneously active proteolytic enzyme and antiprotease activities of the plasmas of patients (a) shortly after admission and usually before therapy is instituted, and (b) at intervals after operation or during therapy.

EXPERIMENTAL

Plasma was separated by centrifuging the oxalated blood of fasting subjects. The plasmas of 3 groups were analyzed: (a) healthy male medical students; (b) patients with about 20 different types of cancer; (c) patients with acute bacterial and virus diseases and a variety of chronic dis-

eases. The diagnosis in each cancer case was confirmed by histologic examination.

The procedure described by Ratnoff (6) for determining chloroform activated plasmin was used except for the initial buffering of the casein substrate. Casein was dissolved in 0.85 per cent saline buffered with M/20 phosphate at pH 7.4.

The spontaneously active proteolytic enzyme is designated as proteolysin in this paper. It is present in the euglobulin precipitate which is obtained by diluting plasma 20 times with distilled water, adjusting the pH to 5.2 with acetic acid and centrifuging. The precipitate representing 2 ml. plasma is dissolved in the buffered saline-phosphate and brought to a 5 ml. volume. The activity of a 2 ml. aliquot is determined after a 1 hour incubation with an equal volume of the buffered casein substrate.

The trypsin inhibitor (antiprotease) is found in the euglobulin supernatant. The details of the procedure for determining this inhibitor are outlined since they represent modifications of a number of methods, particularly those of Christensen. The supernatant is diluted serially with buffered saline at pH 7.4, and 1 ml. aliquots are incubated for 10 minutes with 1 ml. of a standard solution containing 0.02 mg. crystalline trypsin.¹ At the end of this time 2 ml. of 0.3 per cent buffered casein are added and incubated at 37° C. for 15 minutes. Digestion is stopped by adding HCl and sulfosalicylic acid to 1 ml. of the inhibitor digestion mixture according to Ratnoff (6). The turbidity is read in a Klett-Summerson colorimeter. The values for a minimum of 6 different dilutions of a plasma are plotted and a sigmoid curve is obtained. The inhibition unit represents the amount of a plasma which causes a 50 per cent inhibition in the digestion of 0.3 per cent buffered casein by 0.02 mg. crystalline trypsin.

RESULTS

Measurements of the plasmin, proteolysin, and trypsin inhibitor in cancer and miscellaneous dis-

* This investigation was supported by research grants from the National Cancer Institute, U.S. Public Health Service.

¹ Worthington Biochemical Laboratory product containing approximately 50 per cent MgSO₄.

eases are shown graphically in Figure 1. These patients were studied shortly after admission to the hospital. The control ranges represent the limits of variation in 16 normal subjects. The chloroform activated plasmin values of one half of the cancer cases and 37 per cent of the other cases are elevated. The proteolysin values for 30 of the 33 patients with cancer are above normal; the 3 values within the control range are obtained in patients with (a) chronic myelogenous leukemia, (b) multiple myeloma, and (c) a small squamous

tients showing evidence of tissue destruction or inflammation.

All elevated proteolysin activities are plotted against their respective inhibitor values in Figure 2. In all cases in which both the proteolysin and inhibitor values are elevated, well established evidence of tissue breakdown is present. The normal or subnormal inhibition values which are observed in patients with elevated proteolysin activities are difficult to interpret.

The proteolysin and trypsin inhibitor values were determined at various intervals after operation or after instituting therapy in 30 selected patients. The cases are divided into the following 4 groups: (a) partial surgical removal of cancerous

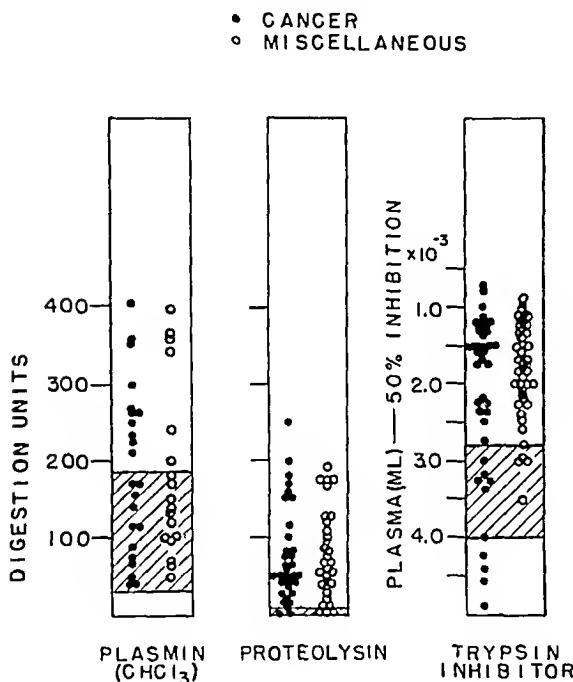


FIG. 1.—Distribution of plasmin, proteolysin and trypsin inhibitor in the plasmas of patients shortly after admission to hospital and before therapy. Hatched areas represent the control ranges.

cell carcinoma of the lip. In the miscellaneous diseases, 26 of the 31 values for proteolysin are elevated; the cases in the normal range represent non-inflammatory disorders. The increased proteolysin activities may be roughly correlated with evidence of tumor necrosis or with inflammatory processes. The trypsin inhibitor values are elevated in 76 per cent of the 39 cancer cases and 90 per cent of the 45 cases of miscellaneous diseases. The 4 subnormal values of the cancer group are seen in 2 cases of multiple myeloma, an early case of carcinoma of the breast and an early untreated case of Hodgkin's disease. A third patient with multiple myeloma has a normal inhibitor value. All elevated inhibitor values are observed in pa-

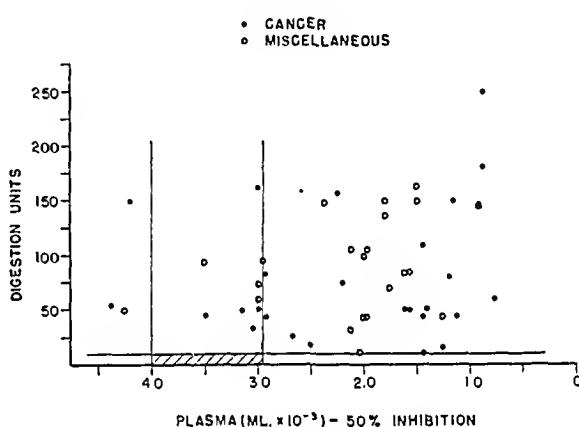


FIG. 2.—A plot of the respective proteolysin and trypsin inhibitor values in plasmas of patients shortly after admission to hospital and before therapy.

tissue; (b) complete surgical removal of the tumor; (c) pneumonia under penicillin therapy; and (d) various chronic diseases. Typical changes observed in each group are shown in Figures 3 and 4.

Partial removal of tumor tissue does not affect the proteolysin activity during the first 3 weeks following surgery. The trypsin inhibitor values increase in these cases (Fig. 3).

The proteolysin activity of the plasma dropped to zero within one week after removal of the urinary bladder in 2 cases of carcinoma of the bladder and after excision of an epidermoid carcinoma of the buccal mucosa in a third patient (Fig. 3). A similar pattern was observed after pneumonectomy for bronchiogenic carcinoma. The values for the 2 cases with initially elevated inhibitor values are not affected, while an initially low value is increased after operation.

The changes in proteolysin and inhibitor in 3 patients with pneumococcal pneumonia treated

with penicillin are shown in Figure 4. It is seen that the proteolysis disappears during the afebrile stage while the inhibitor concentration increases during this period. One patient recovering from pneumonia developed a chill and temperature of 103°F. on the ninth afebrile day. A high proteolysis activity of the plasma appeared within 10 hours following the chill and disappeared 24 hours later when the temperature was normal; the trypsin inhibitor concentration was not affected.

The data for 3 patients with chronic diseases are shown in Figure 4. (a) The edema disappeared and a slight trace of proteinuria was present 2

elevated in practically all patients with malignancy and in the febrile stage of disease. During the course of frequent studies in individual patients, it becomes apparent that the proteolysis is a comparatively sensitive indicator of the effectiveness of treatment or the progress of disease. The protease content of the plasma disappears after successful therapy and remains elevated if the patient does not respond to surgery or drugs. The determination of this enzyme may be a useful guide for determining the progress of a patient's recovery.

The rapid increase in proteolysis concentration probably reflects the presence of a tissue product which is a stimulant to enzyme formation, or perhaps represents an *in vitro* activation of plasminogen. A fairly good correlation between the proteolysis and elevated temperature may be obtained. The elevated values observed in cancer cases are probably associated with tumor necrosis. The removal of the abnormal stimulus by surgery or therapy is accompanied by a rapid disappearance of the proteolysis.

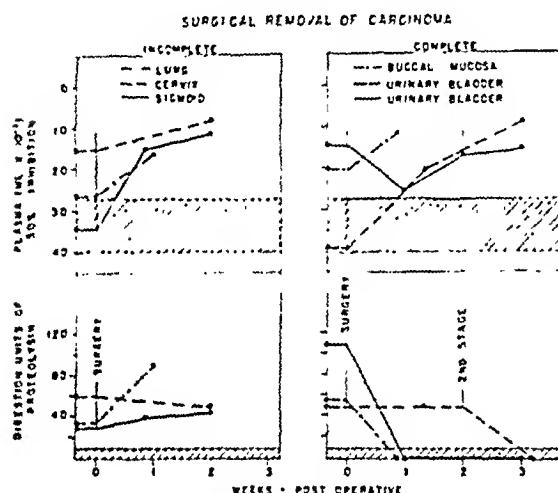


FIG. 3.—The proteolysis and trypsin inhibitor activities in plasmas in patients after surgery.

weeks after tonsillectomy in Miss D who was admitted with glomerulonephritis; at the time of improvement, the proteolysis disappeared, but the inhibitor concentration remained high. (b) A young woman (C) with rheumatic fever (joints) showed no clinical improvement during salicylate administration; both the proteolysis and the inhibitor concentrations remained elevated. (c) A 35 year old man (R) with early miliary tuberculosis was treated with streptomycin and gradually improved during the 4 week period of observation; the proteolysis activity gradually decreased and was no longer present at the end of 3 weeks, but the inhibitor concentration remained elevated.

DISCUSSION

Chloroform activated plasmin is not a reliable measure for the plasma protease content (1, 6). Attempts to correlate the plasmin concentrations with the plasma protease (proteolysis) or with the trypsin inhibitor are unsuccessful.

The proteolysis and trypsin inhibitor values are

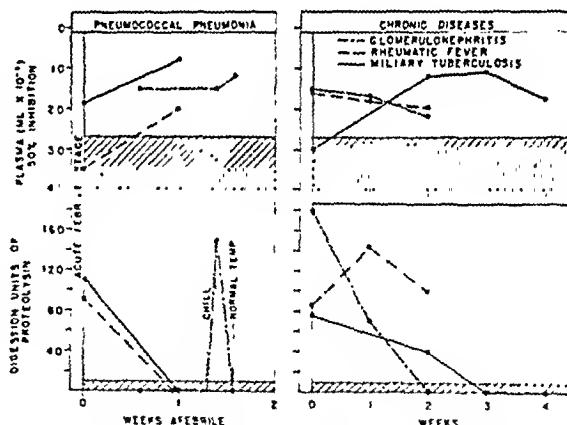


FIG. 4.—The proteolysis and trypsin inhibitor activities in plasmas of patients with pneumonia and chronic diseases.

The trypsin inhibitor concentration increases following acute infections and surgery, but the rise is not as rapid as that seen for proteolysis. The inhibitor returns to the normal range slowly. In 2 patients, one recovering from bronchopneumonia and another recovering from a subphrenic abscess following appendectomy, the inhibitor values returned to the control range 3 weeks after their temperatures were normal. It appears that this inhibitor is associated with the destruction and resolution of tissue.

SUMMARY

The activities of chloroform activated plasmin, spontaneously active protease (proteolysis) and

trypsin inhibitor of the plasma of healthy young men and of patients with cancer and with a variety of acute and chronic diseases were determined.

Observations made on a group of patients shortly after admission to the hospital showed elevated values for the protcolysin and trypsin inhibitor in most cases. No relationship between plasmin, protcolysin, and inhibitor concentrations could be established.

Periodic analyses of the proteolysin and trypsin inhibitor were made on patients (a) after incomplete removal of cancer, (b) after complete removal of cancer, (c) during recovery from pneumonia, and (d) with a variety of chronic diseases. The proteolysin concentration dropped to zero soon after complete surgical removal of a cancer, and in other cases after successful therapy or surgery. The trypsin inhibitor remained elevated for an appreciable period.

The appearance of proteolysin in plasma is associated with necrosis and with inflammatory

processes accompanied by elevated temperatures. The trypsin inhibitor concentration remains elevated in patients who have been operated on and in those whose tissues are undergoing readjustment to the normal state.

The significance of the changes noted in the plasmas of diseased individuals is discussed.

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Antigenic Properties of Nuclei Segregated from Spleens of Normal and Leukemic Mice*

KRISTEN ARNESEN, † YVETTE GOLDSMITH, AND ANNA DEAN DULANEY ‡

(From the Sloan-Kettering Institute for Cancer Research, New York 21, New York)

Various procedures have been described for the segregation of nuclei from mammalian tissues (1, 2, 3, 4, 5, 6). These methods are based on a mechanical breakdown of the cells suspended in aqueous solutions of citric acid, with subsequent separation of the nuclei by centrifugation at different speeds.

In our work with mouse spleens we have investigated other reagents but so far, we have not been able to obtain isolated nuclei without the addition of citric acid. Physiological saline solution is unsatisfactory since the unbroken cells, nuclei, and cytoplasmic granules agglutinate making it impossible to separate these fractions by differential centrifugation. Potassium chloride, of the same molar concentration as the sodium chloride, likewise proved unsuitable.

Various concentrations of sucrose have been used. We have found that nucleoprotein is extracted from the nuclei in both 8.5 per cent (isotonic) and 30 per cent sucrose solutions. When the tissue suspensions were homogenized initially in the sucrose solutions and centrifuged, the sediment was found to be held in a gel which could be dissolved in molar sodium chloride solution. Upon subsequent centrifugation and dilution of the supernate to constitute a 0.14 molar NaCl solution, a fibrous precipitate formed. This stained with methyl green and was therefore presumed to consist of desoxyribose nucleoprotein (7). The addition of very small amounts of citric acid to the sucrose solution prevented the extraction of nucleoprotein, as judged by the above criterion.

Relatively more citric acid was required for the isolation of nuclei from the leukemic than from the normal tissues. A concentration of M/150 to M/125

citric acid, however, proved satisfactory for both types of tissue. Furthermore we have found that the citric acid is necessary only in the first homogenization process. Free nuclei may then be collected in later steps and washed in sucrose solution without apparent loss of nucleoprotein.

The method described here is a modification of the procedure recommended by Doumee (2, 3, 4) which we have adapted to small amounts of tissue. Isotonic sucrose has been substituted for distilled water as a diluent for the citric acid, and the same concentration of sucrose has been used for all washings of the nuclei.

Preparation of Nuclei.—Nuclei were isolated from the spleens of normal and leukemic mice of the AKM stock. The leukemia was a lymphatic strain designated #9421 which was carried by intraperitoneal transfer of leukemic spleen tissue. The animals were killed by cervical fracture, the spleens removed immediately and chilled. To one gram portions of tissue were added 5 ml. of M/125 citric acid in 8.5 per cent sucrose solution. Each portion was homogenized for 5 minutes. This and all subsequent procedures were carried out at temperatures 0 to 5° C.

The homogenates were filtered through gauze and centrifuged for 20 minutes at 600 × g. The supernates were discarded and the sediments suspended in an amount of 8.5 per cent sucrose solution, without citric acid, which was equal to the original volume. Even suspensions were obtained by homogenizing for a few seconds. Subsequently the preparations were centrifuged for 15 minutes at 300 × g., 10 minutes at 200 × g., and 5 minutes at 100 × g. After each centrifugation the sediments were resuspended in the initial volume of 8.5 percent sucrose solution, but without citric acid. Centrifugation for 5 minutes at 100 × g. was repeated until the supernates were clear, or only very slightly opaque, and relatively free of cytoplasmic material, as judged by microscopic examination after staining with Janus Green B.

After the centrifugations were completed, the suspensions were allowed to sediment in narrow cylinders for 30 minutes. Most of the remaining unbroken cells settled out on the bottom of the cylinder. The upper 95 per cent of the preparation was aspirated and the sedimentation in the narrow cylinders repeated 2 or 3 times.

* This study was supported by grants from the Office of Naval Research and the James Foundation of New York, Inc.

† Present address: American-Scandinavian Foundation, Permanent Address: Institute of Pathology, University of Oslo, Norway.

‡ Permanent address: Institute of Pathology, University of Tennessee, Memphis, Tenn.

Microscopic examination of the final suspensions, stained with Janus Green B, indicated that the nuclei retained their typical morphologic characteristics and in general, were free of cytoplasmic material. However, some of them showed minute cytoplasmic tags.

The nuclei were then counted in a haemocytometer and the suspensions diluted to 200 million nuclei per 1 ml. and distributed in ampoules which were stored at -20° C.

The desoxyribose nucleic acid (DNA), pentose nucleic acid (PNA), and nitrogen (N) contents of each of these preparations were determined.¹ The results are given in Table 1.

TABLE 1

SHOWING THE CONTENT OF DNA, PNA AND N IN MGM/ML AS WELL AS THE PNA/DNA RATIO IN THE SUSPENSIONS OF NORMAL AND LEUKEMIC SPLEEN NUCLEI

	DNA	PNA	PNA/DNA	NITROGEN
				GEN
Normal nuclei	1.316	0.098	0.07	1.008
Leukemic nuclei	1.246	0.411	0.33	1.116

It is apparent that the DNA and N values of the normal and leukemic spleen nuclei suspensions do not differ significantly. In contrast the PNA of the leukemic nuclei is more than 4 times that of the normal. In general, these quantitative differences between the PNA values of the normal and leukemic spleen nuclei are in accordance with those reported by Petermann, Alfin-Slater, and Larack for the "nuclear fraction" (8).

Serologic Studies.—The normal and leukemic nuclei suspensions were diluted to contain 0.1 mg. nitrogen in 1 ml., and injected intravenously into 6 rabbits. Six injections of 1 ml. were given to each animal at intervals of 2 or 3 days. One week after the last injections the rabbits were bled and the sera of each group used for serologic tests.

Complement fixation tests were carried out according to the technique described in a previous paper (9). For the tests the nuclei suspensions were diluted to a nitrogen content of 0.0055 mg. per ml. Thus the nitrogen values of the nuclei antigens used for immunizing purposes and in complement fixation tests were the same as those of the cytoplasmic fractions, *i.e.*, the M (mitochondria) and P (submicroscopic particles) (10). This was done in an effort to compare the antigenic properties of the various cell fractions as standardized by nitrogen content. The results of the complement fixation tests are given in Table 2.

As shown in Table 2 the nuclei preparations proved to be poor antigens in that they stimulated the development of anti-nuclei antibodies of low

¹ The nucleic acid analyses were carried out in Dr. M. L. Petermann's laboratory by Miss A. M. Larack. The nitrogen analyses were done in Dr. G. B. Brown's laboratory by Mr. R. C. Funk, Jr.

titors. No serologic specificity was demonstrated since both the normal and leukemic antisera exhibited almost identical titers for the normal and leukemic nuclei antigens. Interestingly both of the anti-nuclei sera gave cross reactions with the cytoplasmic fractions from both normal and leukemic spleen which were of higher order than those obtained with the nuclei antigens. The titers for the cytoplasmic antigens were 2 to 4 times those for the nuclei antigens. In contrast the titers of the anti-cytoplasmic sera for the nuclei antigens were significantly lower than those obtained for the homologous M and P fractions segregated from spleen cells.

Pollister and Leuchtenberger (11) have recently pointed out that considerable amounts of non-histone protein and nuclear enzymes are lost during the procedure of isolating nuclei. We recognize the possibility that the antigenic properties of nuclei preparations may be modified during any isolation process. There is also evidence that nucleoproteins *per se* are poor antigens (12). Maculla (12) shows that the antisera to spleen nucleoproteins gave substantially higher titers with the homologous residue and extract fractions than with the nucleoprotein antigens.

Our data from serological tests therefore suggest one of the following conclusions: a) Nuclei pre-

TABLE 2

SHOWING THE TITERS OF THE ANTI-SERA TO THE VARIOUS SPLEEN CELL FRACTIONS FOR THE NUCLEI AND CYTOPLASMIC ANTIGENS

Anti-SERA	CELL FRACTION ANTIGENS					
	Normal nuclei	Leuk. nuclei	Nor. mito.	Leuk. mito.	Nor. P-1	Leuk. P-1
Normal nuclei	40	40	160	80	160	160
Leukemic nuclei	80	80	160	160	320	320
Normal mitochondria	80	80+	640	640+	640	640
Leukemic mitochondria	80	80+	640	1280	640	640
Normal P-1	40	40+	80	160	80	160
Leukemic P-1	40	80	160	320	160	320

pared according to a modified Dounce method are poor antigens as compared with the cytoplasmic particles of the same cell, and judged by the titers of antibodies elicited; b) The cross reactions exhibited between the anti-nuclei sera and cytoplasmic fractions, as well as those occurring between the anti-M and anti-P sera and nuclei antigens, are either due to "contaminated antigens" or to sharing of antigenic components by the cytoplasmic and nuclei fractions. By "contaminated antigens" we refer to the possible adsorption of nuclear materials on the M and P particles during the segregation process, or of cytoplasmic particles on nu-

der. The possibility that the nuclei might carry residual cytoplasmic tags must also be considered. Adsorption of the anti-nuclei sera with mitochondria did not reduce significantly the titer for these antigens in subsequent complement fixation tests. This suggests that the nuclei were not grossly contaminated with cytoplasmic constituents which could have explained the formation of anti-cytoplasmic antibodies. Whatever the explanation, it is evident that the cytoplasmic elements of the cell are the dominant antigens.

SUMMARY

Nuclei have been isolated from spleens of normal and leukemic mice by use of a modified Dounce procedure. These nuclei are relatively pure as judged by morphologic and staining criteria. Such nuclei preparations are poor antigens in that very low titers of antibodies to nuclei are produced upon immunization of rabbits. Antigenic specificity was not demonstrated through use of the complement fixation test. Cross reactions between the nuclei and cytoplasmic fractions were demonstrated in complement fixation tests. Cytoplasmic fractions from the same spleen cells were found to be superior antigens.

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A Correlation of Fluorescence of Human Urine with Benign and Malignant Growth

HAROLD M. RABINOWITZ, M.D.

(From the Research Division, Department of Surgery, Maimonides Hospital, Brooklyn, New York)

In 1869 Jaffe (1) noted that normal urine exhibited a blue-green fluorescence on exposure to ultraviolet radiation. Subsequently attempts were made to investigate the relation between the output of fluorescent substances and that of other urinary constituents in human urine. In this connection Squires and Jeffree (2) investigated the fluorescence of urine by means of a simple form of ultraviolet fluorophotometer. Within certain limits of dilution, the intensity of fluorescence is proportional to the concentration of the fluorescent substance. Based upon this principle, a series of extended observations on eight individuals was completed. The results indicated that the daily output of fluorescent material was independent of the volume of urine excreted and bore no close relation to that of the familiar urinary constituents.

The purpose of this communication is to report the studies of fluorescent intensity of a blue fluorescent substance and a red fluorescent substance respectively present in human urine, their relation to each other, and their significance in normal, benign, and malignant growth. Values of fluorescent intensity of the blue fluorescent substance and the red fluorescent substance will be expressed as galvanometer readings (arbitrary units) and designated as B values for the blue fluorescent factor and R values for the red fluorescent factor respectively, and their relation to each other as B:R ratio. The B and R values and B:R ratios on samples of 24 hour urines were determined on three groups of cases: a). Controls comprising cases free of benign or malignant growth in good health and those free of benign or malignant growth with associated ailments; b). Proven cases of benign growth including pregnancy; c). Proven cases of malignant growth.

PROCEDURE

Apparatus: Phaltz & Bauer Fluorophotometer, Corning Red Filters #2412, Corning Blue Filters #5543, glass cuvettes, glass standard B₁, 85 watt mercury capillary arc lamp AH3 with light range 3100 to 4200 millimicrons, ultra violet filter #5840.

Ingestion of drugs and vitamins was avoided

for 48 hours previous to collection of 24 hour urine. Receptacle for collecting urine was washed clean with warm water exclusively, as soaps contain fluorescent substances. Cases with markedly impaired kidney function, liver damage, and jaundice were precluded. Urine containing pus or otherwise unduly turbid was also precluded. Collected urine was kept in a cool place or refrigerator.

Total amount of urine collected over a period of 24 hours in this series of cases was 950 cc. \pm 25 cc. It was thoroughly mixed, and a 4 ounce sample was taken for assay. Seventeen c.c. of the clear supernatant fluid of the 4 ounce sample was poured into a rectangular cuvette, the capacity of which is 20 c.c. The glass standard B₁ and the Corning Blue Filter #5543 were inserted into provided rectangular slots. By means of a lever with scale and indicator connected with the iris diaphragm, light intensity was adjusted to a reading of 50 on the galvanometer scale. The glass standard B₁ was then removed, and replaced by the cuvette containing the clear supernatant fluid and a reading was taken on the galvanometer scale for blue fluorescent intensity. The cuvette was then removed and replaced by the glass standard B₁ and the light intensity again adjusted to a reading of 50 on the galvanometer scale, following which the glass standard B₁ and the Corning Blue Filter were removed from their respective slots and replaced by the Corning Red Filter #2412 and the Cuvette containing the sample of clear supernatant fluid, following which a reading was taken on the galvanometer scale. Light intensity adjustment was made for each determination. Strict attention to cleanliness of the filters and cuvettes is paramount before determinations are undertaken.

RESULTS

Controls.—The control group comprised 150 cases of which 100 enjoyed good health and were free of benign or malignant growth and 50 cases were free of benign or malignant growth with associated common ailments. The results of studies of the R values (red fluorescent substance) in the control group (Fig. 1) indicated a maximal value

for the R factor of 2.0. The values for the B factor (blue fluorescent substance) covered a wide range of flux and the result of the studies of the relation of the B values to the R values in this group indicated a minimal B:R ratio of 6.0:1.0. Associated ailments did not alter the R and B values to any significant degree. In a group of 50 cases in the control group (Fig. 1) the R values were below 1.5 and the B values correspondingly low with a preponderance of B:R ratios of 6.0:1.0. The significance of the above values and of the minimal B:R ratio of 6.0:1.0 in the control group will be clarified when compared with the R and B values and the

maximal R value of 2.0 indicated in the control group, and the B values were outstandingly higher than those of the control group with resultant B:R ratios significantly greater than those of the control group. In a group of 21 pregnancy cases (Fig. 1) the R values were significantly raised above the optimal R value of 2.0, indicated in the control group and the B values were proportionately raised with resultant higher B:R ratios than the control group, thus paralleling the benign growth group. Samples of amniotic fluid from 10 normal delivery cases presented R values markedly raised above 2.0, (the maximal value of the control

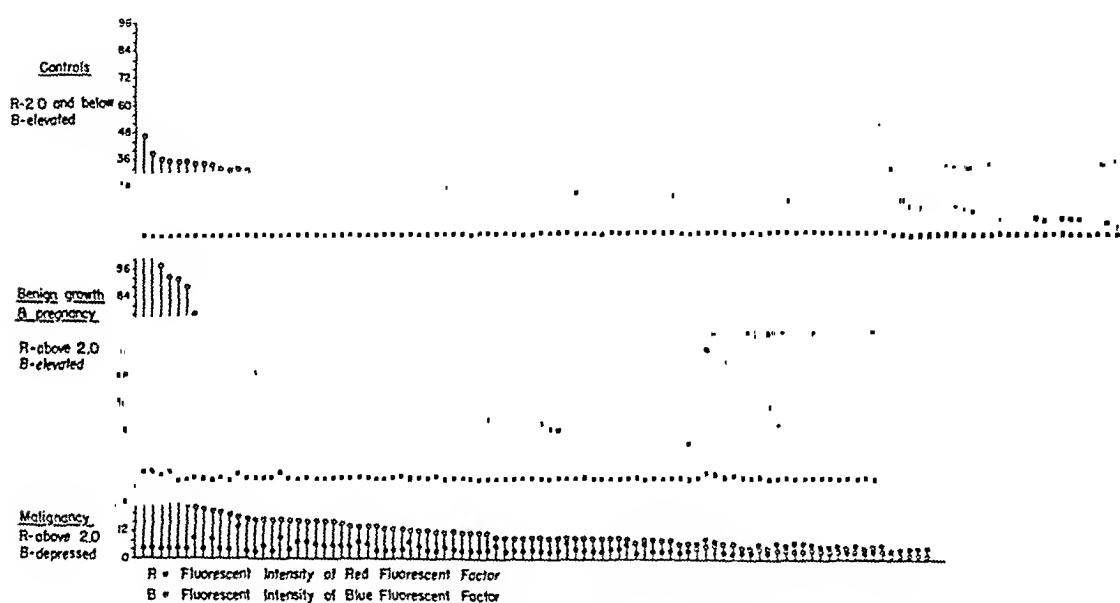


FIG. 1.—Comparative R and B values in normal, benign growth including pregnancy and malignancy

B:R ratios of the benign and malignant growth groups respectively. Additional determinations for R and B values and B:R ratios were made upon a group of 20 cases, ranging over a period of from 2 to 14 years following radical resections for malignant lesions. All enjoyed good health, with no evidence of local recurrence or metastasis. The R and B values and B:R ratios paralleled the control group.

Benign growth and pregnancy.—In a group of 91 cases (Fig. 1) with proven benign growths comprising fibroid uterus, adenomas and polyps of the gastrointestinal tract, uterine polyps, hypertrophied prostate, fibroadenomas of the breast, cystic mastitis, benign tumors of the ovary, endometriosis, benign tumors of bone, pituitary adenoma, and thyroid adenomas, the R values were consistently and significantly raised above the

group), and the B values were correspondingly raised with B:R ratios paralleling the benign growth group. The presence of R and B factors in amniotic fluid with values paralleling the benign growth group aroused considerable interest as the urinary constituents of amniotic fluid are shared by both the fetal kidney and placenta and was likewise singularly revealing since the placenta acts as a barrier to most foreign substances. The elevated R values above the indicated maximal value of 2.0 of the control group and the proportionate and consistently raised B values and B:R ratios were outstanding in both benign growth group and pregnancy group.

Malignancy.—In a group of 101 cases with proven malignant growth (Fig. 1) comprising malignancies of the gastrointestinal tract, lung, thyroid, uterus, prostate, ovaries, breast, bone,

acute lymphatic leukemia and lymphosarcoma, the results of the studies indicated that the R values were strikingly raised above the maximal value of 2.0 of the control group, whereas the B values were strikingly depressed with resulting low B:R ratios, less than 5.0:1.0, the indicated minimum B:R ratio of the control group being 6.0:1.0.

Relation of R and B values to altered growth.—The indicated maximal level of the R factor in the control group was 2.0, whereas in benign growth, pregnancy and malignant growth, the level of the R factor was consistently and significantly elevated above 2.0. The B values in the control group were correspondingly raised or lowered proportional to the level of the R values. For example,

ratios of the control, benign growth and pregnancy group.

Table 1 presents a comparative tabulation of the R and B values and B:R ratios of cases affected with benign and malignant growths respectively, in homologous organs, and clearly indicates that the contrasting R and B values and B:R ratios in benign and malignant growth respectively follow a definite and similar pattern regardless of the type of organ affected.

On the basis of approximately 1500 determinations of R and B values and B:R ratios on 400 cases, comprising normal, benign including pregnancy and malignant growths, the results of the data indicated the following: in the control group, a minimal B:R ratio of 6.0:1.0 and a maximal R

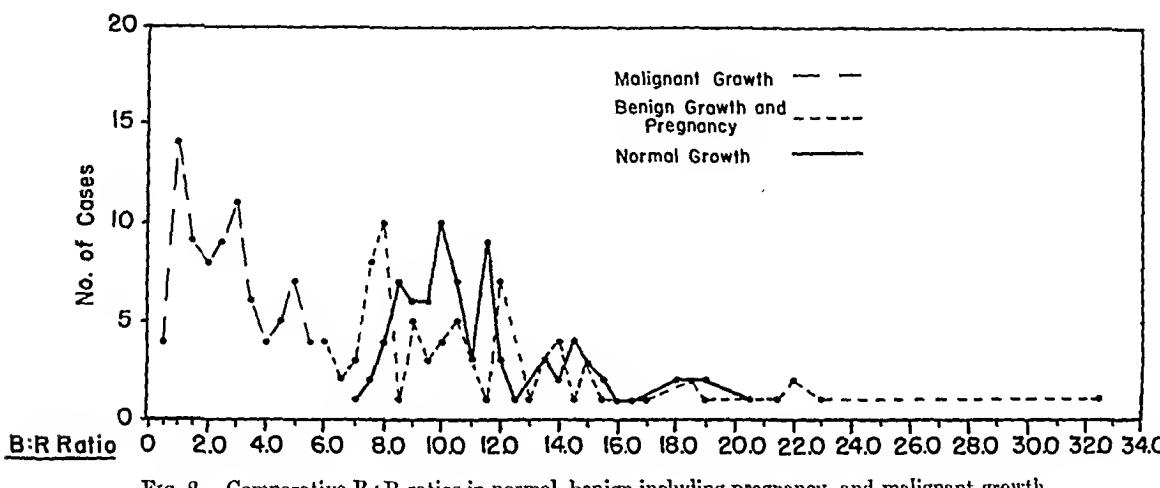


FIG. 2.—Comparative B : R ratios in normal, benign including pregnancy, and malignant growth

in a group of 50 cases in the control group (Fig. 1) the R values were below 1.5 and the B values were correspondingly low, while in the remaining cases of the control group, in which the R values ranged from 1.5 to a maximum of 2.0 the B values indicated correspondingly greater levels proportional to the values of the R factor. Likewise the B values were markedly elevated in the benign and pregnancy group (Fig. 1) proportional to the levels of the R values which were consistently elevated above 2.0. In striking contrast, the B values in the malignant growth group (Fig. 1) were markedly depressed though the R values were elevated resulting in a depressed B:R ratio of less than 5.0:1.0, the indicated minimum B:R ratios of the controls, benign growth and pregnancy being 6.0:1.0.

Figure 2 represents a plotted graph of the B:R ratios of controls, benign growth including pregnancy, and malignant growth groups and indicates graphically the low B:R ratios of the malignant growth group in contrast to the elevated B:R

value of 2.0; benign growth group including pregnancy, a minimal B:R ratio of 6.0:1.0 and R values greater than 2.0; malignant growth group, B:R ratios less than 5.0:1.0 and R values greater than 2.0.

Diagnostic accuracy.—Neoplastic diseases of known low grade of magnitude comprising 4 cases of Hodgkin's disease, 2 cases of chronic lymphatic leukemia, 1 case each of reticulum cell tumor and giant cell sarcoma presented R and B values and B:R ratios within the range of the control group.

In the course of routine determinations, 6 cases of cirrhosis of the liver with liver damage confirmed by liver function tests and blood chemistries, and free of malignancy, presented high R and low B values and B:R ratios less than 5.0:1.0 which are within the range of the malignant growth group. The altered R and B values and B:R ratios reverted to values within the range of the normal or benign growth groups after appropriate treatment for cirrhosis, such as high protein

and low fat diet. Two cases of infectious hepatitis with reversal of albumin globulin ratios and evidence of liver damage presented B:R ratios of less than 5.0:1.0 similar to that in the malignant growth group. They reverted to values within the range of the control or benign growth groups following appropriate treatment. Two cases of severe toxemia of pregnancy and preeclampsia with liver damage and reversal of albumin globulin ratio presented B:R ratios of less than 5.0:1.0, and elevated R values similar to those of the malignant growth group, and reverted to benign growth group values following emptying of the uterus. Apparently, altered R and B values and B:R ratios within the range of the malignant growth group are associated with liver damage in cirrhosis of the liver, infectious hepatitis, and severe toxemia of pregnancy and are reversible when amenable to appropriate treatment. In the proven malignant growth group the R and B values and B:R ratios are irreversible except by radical surgery or radiation. It may be pointed out that liver damage was the common outstanding factor in all these cases. In a small group of 10 cases one can hardly make deductions or come to any definite conclusions. Studies of liver function tests in liver damage associated with cirrhosis, infectious hepatitis, toxemia of pregnancy and other forms of liver damage with parallel determinations of R and B values and B:R ratios on a large group of cases may shed some light on the mechanism by which R and B values and B:R ratios are altered in liver damage. Such values could only be determined at this time in the absence of jaundice.

In the follow up of 22 cases over a period of one year following radical surgery for widely diversified malignant lesions, a few observations of interest were noted. For example, R and B values and B:R ratios within the range of the malignant growth group preoperatively, persisted post-operatively as long as four to eight weeks before the values reverted to those of the control group. In others, benign growth group values preceded the ultimate reversion to control group values post-operatively and in two cases of subtotal gastrectomy for adenocarcinoma of the stomach, post-operative R and B values and B:R ratios similar to those of the normal or control group persisted for 12 months and 20 months respectively, during which time they enjoyed good health and subsequently presented elevated R values and depressed B values and low B:R ratios with accompanying clinical and x-ray evidence of metastases and local extension.

From these observations, one is inclined to assume that the pattern of the R and B values and

B:R ratios parallel the postoperative clinical course and indicate the reversion of malignant values to the normal and similarly, the transition from the normal to autonomous invasive growth. Greenstein (3) reported a most comprehensive and painstaking comparative study of enzyme activity in normal tissue on the one hand and completely malignant tissue on the other, and stated that, "The intermediate forms have not been studied as yet because it is not known just at what point the malignant process intervenes." A planned statistical study of the R and B values and B:R ratios in samples of 24 hour urines may lend itself to the

TABLE I
COMPARATIVE R AND B VALUES AND B:R RATIOS IN BENIGN
AND MALIGNANT LESIONS AND B:R RATIOS IN BENIGN
HOMOLOGOUS ORGANS

	Benign			Malignant	
	R	B	B:R Ratio	R	B
<u>Stomach</u>	3.0	29.0	9.66 : 1	4.2	2.6
	2.0	17.0	8.5 : 1	6.0	4.0
	3.0	23.8	7.9 : 1	5.0	11.0
	2.0	21.2	10.6 : 1	2.0	3.8
	2.0	32.5	16.2 : 1	6.2	8.0
<u>Prostate</u>	3.0	24.0	8.0 : 1	6.0	17.0
	2.2	29.1	13.2 : 1	4.0	9.5
	3.0	19.4	6.4 : 1	3.0	8.6
	3.0	97.0	32.3 : 1	3.0	8.0
	2.6	20.6	7.9 : 1	6.0	17.0
<u>Breast</u>	3.6	64.0	17.7 : 1	3.8	11.0
	3.0	31.9	10.6 : 1	7.0	18.0
	4.0	60.0	15.0 : 1	4.0	20.0
	2.4	30.0	12.5 : 1	6.2	8.0
	2.2	23.2	10.5 : 1		
<u>Uterus</u>	3.0	31.4	10.4 : 1	4.5	9.0
	3.0	27.0	9.0 : 1	7.6	12.0
	3.0	37.6	12.5 : 1	3.0	8.4
	3.8	59.0	13.1 : 1	3.0	15.0
	4.2	76.5	18.2 : 1	6.6	24.6

study of the phases of transition from normal to ultimate autonomous invasive growth in the human and may throw some light on the concept offered by Ivy (4), "The benign cell or tumor represents an arrested stage of a potentially malignant process."

COMMENT

Though conclusive deductions cannot be made until a larger series of cases has been evaluated in parallel with other approaches to the study of the growth mechanism, nevertheless, the results of these studies indicate that an inadequate B factor, as represented by a low B value in the face of an elevated R value is a constant and persistent finding with the advent of autonomous invasive growth anywhere in the body. In this connection it is of particular interest to point out that in a

contribution on the relationship of pyrrol compounds to carcinogenesis, Figge (5) referred to three hypothetical factors in his hypothesis of the sensitization of the cell to malignant transformation, in which the role of the red fluorescent porphyrin was stressed. It has indeed opened up a new field of interest in the study of fluorescent porphyrins as related to the genesis of cancer. The data presented in this communication is not submitted as a test for cancer but rather as a study of the possible role of the red and blue fluorescent substances respectively with relation to the mechanism of growth and its alterations.

SUMMARY

Values of fluorescent intensity of a blue fluorescent substance and a red fluorescent substance respectively, were determined on samples of 24 hour urines in 400 cases, comprising controls, benign growth, pregnancy and malignant growth groups. Values were expressed as galvanometer readings (arbitrary units) and designated as B values and R values for the blue fluorescent and red fluorescent factors respectively and their relation to each other as B:R ratio. The B and R values and B:R ratios

in the controls, benign growth including pregnancy and malignant growth groups were evaluated and correlated. Determinations for R and B values and B:R ratios were made upon 20 cases following radical surgery from 2 to 14 years. A group of 22 cases on whom radical surgery was performed for a wide range of malignant lesions were followed up throughout the year 1948 with frequent determinations of the R and B values and B:R ratios and correlated with the changes in the clinical course, such as extension of malignancy and advent of metastases.

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The Distribution and Action of a Radioactive Oxazine Dye in Tumor-Bearing Mice*

HENRY A. SLOVITER, PH.D., M.D.

(From the Harrison Department of Surgical Research, School of Medicine, University of Pennsylvania and the Department of Neurosurgery, Hospital of the University of Pennsylvania, Philadelphia, Pa.)

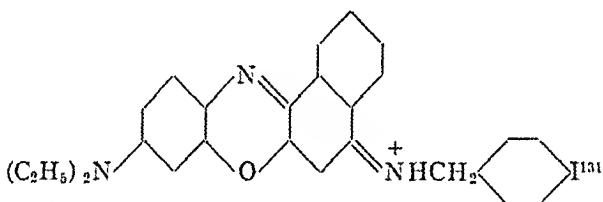
Dyes have long been used in investigations of cancer with the hope of developing useful diagnostic and therapeutic agents. The aim in such investigations has been to find a dye which, in the intact animal, stains living tumor cells without staining surrounding tissue. A large number of acid dyes, especially in the azo series, has been rather intensively investigated for such a purpose. From these investigations there has resulted general agreement (1, 11) that acid dyes do not localize in viable tumor cells but accumulate in the stroma of the tumor.

The incorporation of a radioactive isotope into a dye molecule has been achieved with several acid dyes (5, 10). Studies of the distribution of radio-

and intense staining of viable tumor cells when administered orally or parenterally. The halogen derivatives of Nile blue 2B were prepared (8) and found to give good staining of tumor cells in mice. The radioactive iodine derivative of Nile blue 2B has been prepared (9). In the present work, this radioactive dye has been administered to animals. It is the intent of this paper to describe these experiments and their results.

MATERIALS AND METHODS

Radioactive Dye.—The dye used is a radioactive iodine derivative of Nile blue 2B. The iodine is organically bound and is not in an ionizable state. The structure and nomenclature of the dye are:



bromo derivatives of trypan blue and Evans blue (5) and of a radioiodo derivative of trypan blue (10) in tumor-bearing mice have been reported. Radioactive diiodofluorescein (6) has been used for the localization of intracranial neoplasms. No results of any direct evaluation of therapeutic activity of these substances have been reported.

It has been shown (3, 4) that a basic oxazine dye, Nile blue A (Colour Index number 913), when administered orally to tumor-bearing mice causes diffuse staining of viable tumor cells without staining of the tumor stroma or the surrounding tissues. This staining action is accompanied by a marked retardation of the rate of growth of the tumor. Other closely related oxazine dyes were similarly tested. Of these tested, Nile blue 2B (Colour Index number 914) was found to give most uniform

5-(p-radioiodobenzylamino)-9-diethylaminobenzo[a]phenoxazine

The preparation of this radioactive dye has been described in detail in a previous publication (9). The specific activity of the dye at the start of this investigation was 0.55 millicurie per gram. For injection purposes, an 0.1 per cent solution of the dye in 5 per cent glucose was prepared by adding the dye to sterile glucose solution, boiling gently for 20 minutes, and filtering into a sterile flask.

Animals Used and Method of Administration of Dye.—Inbred mice of the C3H strain were used. The mice were implanted subcutaneously in the right axillary region with either of two tumors native to the strain. The tumors used were a fibrosarcoma (methylcholanthrene induced) and a transplantable spontaneous mammary carcinoma. These tumors have been transplanted through

* Aided in part by a grant to Dr. Margaret R. Lewis from the National Cancer Institute.

many generations with no incidence of spontaneous regression.

The dye was administered by either of two methods. It was administered orally by mixing it into their food to the extent of 0.4 per cent of the weight of the food. It was administered parenterally by subcutaneous injection of the 0.1 per cent solution. The average dose was 0.4 ml. The injection was made on the side of the mouse opposite to that bearing the tumor.

In one experiment the radioactive dye solution was administered intravenously to a normal dog in daily doses of 25 ml. for five days. This experiment was performed for two reasons. First, it was

TABLE 1
DISTRIBUTION OF RADIOACTIVITY
IN A NORMAL DOG

Tissue	Counts per minute (measured)	Counts per minute per gram (corrected for decay)
Liver	176	16
Spleen	93	5
Kidney	29	4
Muscle	11	1
Blood	9	41
Bile	722	1000
Brain	4	0.6
Thyroid	19	63
Lung	29	6

of interest to determine the pattern of distribution of radioactivity in an animal more comparable to the human than is the mouse. Secondly, it is readily possible in the dog and not in the mouse to take a sample of thyroid tissue for radioactivity measurement.

Measurement of Radioactivity in Tissues.—Immediately after sacrificing the experimental animal, weighed portions of various tissues were taken for radioactivity measurement. These were prepared for measurement by either of two methods. One method was to extract the minced tissue with methanol acidified with acetic acid and evaporate the filtered extract to dryness in a 40 mm. open Petri dish. Extraction by this technique was sometimes incomplete since measurable radioactivity was present in the tissues after extraction. A more reliable method for preparing the tissues for radioactivity measurement involved digestion of the weighed sample with concentrated nitric acid to which a little silver nitrate solution had been added. The digestion mixture was evaporated to a small volume in a 40 mm. open Petri dish prior to measurement.

The radioactivity of these preparations was measured with a thin window Geiger-Müller counter.

EXPERIMENTAL AND RESULTS

Distribution of Radioactivity in a Normal Dog.—One normal dog weighing 9.3 kilograms received intravenously 25 ml. of 0.1 per cent radioactive dye solution daily for five days. No untoward effects were noticed during this period and three hours after the last injection the dog was sacrificed. Autopsy showed that all organs were grossly and microscopically normal. The radioactivity found in the extracts of the various tissues is shown in Table 1. The data of Table 1 show very high radioactivity in the bile, indicating that this is the principal avenue of excretion of the dye. The relatively high radioactivity in the thyroid is probably due to release of iodine in the metabolism of the dye since the thyroid gland is not visibly stained by the dye. Other organic iodine compounds such as those used for cholecystography (*e.g.* Priodax), are known (7) to significantly affect thyroid function possibly through release of iodine in metabolism. The very low radioactivity in the brain and the absence of staining of the intact central nervous system are of significance in connection with the possible use of the radioactive dye for the localization of intracranial neoplasms.

Distribution of Radioactivity in Tumor-bearing Mice.—The distribution of radioactivity in tumor-bearing mice which received a single injection of radioactive dye was studied. The level of radioactivity of the various tissues varied from mouse to mouse depending on the quantity of dye administered and the time intervals involved. In the pres-

TABLE 2
DISTRIBUTION OF RADIOACTIVITY IN
TUMOR-BEARING MICE

Tissue	2.5 hours after injection		24 hours after injection counts/min.
	counts/min.	counts/min./g.	
Tumor	209	696	0
Liver	254	508	0
Spleen	124	477	0
Kidney	206	824	0
Blood	488	976	0
Muscle	58	193	0
Feces			614

ent study the absolute values of the radioactivity were considered to be of relatively little import, the principal objective being to determine the relative radioactivity in the various tissues. In Table 2 are listed typical results obtained with C3H mice bearing transplanted fibrosarcomata which received a single subcutaneous injection of 0.9 ml. of the 0.1 per cent solution of radioactive dye. The mice were sacrificed at 2.5 hours and at 24 hours following injection and the tissues were digested with acid and silver nitrate prior to measurement of radioactivity.

The data of Table 2 show that all of the radioactive dye is excreted within 24 hours; in part *via* the kidney and probably to a greater extent *via* the bile into the intestine. The data further show that when the blood level is high, the concentrations in the liver, kidney and spleen are of the same order of magnitude as that in the tumor. The concentration of dye in muscle is considerably lower than in the tumor.

Distribution of Radioactivity in Tumor-bearing Rats.—The radioactive dye was also administered to a small number of inbred rats bearing transplanted fibrosarcomata. The staining of tumors in the rat is not as good as in the mouse. The limited data obtained with rats showed that the concentration of dye (from radioactivity measurements) was considerably higher in the liver than in the tumor. This species difference may be related to the fact that the rat has no gallbladder whereas the mouse does.

Effect of Oral Administration of Radioactive Dye on Survival of Tumor-bearing Mice.—A group of adult C3H mice were implanted subcutaneously with mammary carcinomata from a tumor native to the strain which had been transplanted through several generations. No cases of spontaneous regression had been observed with this tumor. On the thirteenth day after implantation of the tumors, the radioactive dye was administered orally (mixed with the food as 0.4 per cent of the weight of the food). The tumors were definitely palpable and growing at the time the feeding of the dye was started. The administration of the dye was continued for approximately 17 days and then the mice were given ordinary food until they died. Survival time was computed as the number of days from time of implantation of tumor to death.

Another group of adult C3H mice implanted with the same mammary carcinomata served as a control group. They were prepared and fed in the same manner as the previous group except that the dye used was prepared in identical fashion with ordinary iodine instead of radioactive iodine. The data for these two groups is shown in Table 3.

Although the number of animals involved in this experiment is small, a marked difference in the average survival time of the two groups (74 days for the radioactive group, 36 days for the control group) is shown. This indicates that the radioactivity carried to the tumor is a significant factor in prolonging the life of these tumor-bearing mice.

Mice of this same strain implanted with this same tumor which receive no dye survive an average of 25 to 30 days (2). Thus, in the radioactive dye two factors are operative in prolongation of

life: the retardation of tumor growth by the dye and the radiation effect of the radioactive isotope.

During the period of treatment with the radioactive dye and until just before death, the mice remained active and in good physical condition. The tumors grew slowly and in two of the mice treated with radioactive dye, there was a temporary but marked diminution in the size of the tumor.

Effect of Parenteral Administration of Radioactive Dye on Survival of Tumor-bearing Mice.—Ten adult C3H mice were implanted subcutaneously with fibrosarcomata and the mice were untreated

TABLE 3

ORAL ADMINISTRATION OF RADIOACTIVE DYE TO
MICE WITH MAMMARY CARCINOMATA

RADIOACTIVE DYE GROUP Days dye fed	Days survived	CONTROL DYE GROUP Days dye fed	Days survived
17	58	13	27
17	58	15	28
18	62	16	29
17	83	18	37
18	90	18	44
14	92	14	50
Ave: 74		Ave: 36	

TABLE 4

PARENTERAL ADMINISTRATION OF RADIOACTIVE DYE
TO MICE WITH FIBROSARCOMATA

Period of dye injections (days)	No. of injections	Days survived
17	12	37
17	10	48
17	12	48
17	9	36
16	8	33
16	9	62
16	7	94
16	7	57
16	7	62
16	8	37
Ave: 9		Ave: 51

until approximately 2 weeks after implantation at which time the tumors were moderately large. The mice then received subcutaneous injections of 0.4 ml. of the radioactive dye solution on an average of every second day. This treatment was continued for about 16 days following which the mice were untreated. Survival times were recorded. The data are summarized in Table 4.

The survival time of untreated mice bearing the tumor used in this experiment is approximately 20 days (2). The average survival time of the treated mice shown in Table 4 is 51 days, a significant increase over the untreated. In several of the treated mice in this series there was partial sloughing of the tumor and comparatively little viable tumor

tissue was present at death. In the case of the mouse which survived 94 days, most of the tumor had sloughed and there remained only a very thin rim of tumor tissue surrounding a small amount of caseous necrotic material.

SUMMARY

1. The radioactive iodine derivative of the oxazine dye Nile blue 2B has been administered to a normal dog and to tumor-bearing mice. The distribution of radioactivity in the tissues of these animals has been determined. In the dog, the radioactivity is very high in the bile and rather high in the thyroid gland. In the tumor-bearing mice, the concentration of dye in the tumor is of the same order of magnitude as in the liver, kidney and spleen.

2. The administration, either orally or parenterally, of this radioactive dye to tumor-bearing mice has been shown to result in marked prolongation of life of the mice. The prolongation of life by the radioactive dye has been shown to be significantly greater than by the same non-radioactive dye.

ACKNOWLEDGMENTS

The author is grateful to Dr. Margaret R. Lewis of The Wistar Institute of Anatomy and Biology for preparing and supplying the rats and mice used in this investigation and for her continued interest in the work. Dr. Milton H. Paul and

Mr. Bernard Shapiro kindly assisted with the radioactivity measurements. The encouragement and support of the present work by Dr. I. S. Ravdin and Dr. F. C. Grant are greatly appreciated.

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The Evaluation of Diagnostic Tests for Cancer

II. Inhibition of Serum Alkaline Phosphatase by Zinc Ion (The Roche Test)*

WILLIAM H. FISHMAN, A. WAYNE, AND F. HOMBURGER

(From the Cancer Research and Cancer Control Unit of the Department of Surgery and the Departments of Medicine and Biochemistry, Tufts College Medical School, Boston 11, the Division of Clinical Research of the Jewish Memorial Hospital, Roxbury, and the Cancer Research Laboratories of the Holy Ghost Hospital, Cambridge, Massachusetts)

The history of diagnostic tests for human cancer has recently been critically reviewed by Homburger (1). Criteria were given for making decisions with regard to the suitability of various proposed tests for malignancy. One of these, the Roche test, had been selected for study and evaluation some time ago (2).

Roche *et al.* (3, 4, and 5) reported that in the presence of zinc ion in a concentration of $1 \times 10^{-5} M$ the serum alkaline phosphatase was specifically inhibited in cancer patients. The series included 16 normal subjects, 45 non-cancer patients and 142 cancer patients. Attention was directed to the high zinc concentration in tumors as an explanation of the specificity of the zinc reaction in cancer patients. Following surgical removal of the tumor, the serum of cancer patients was found to revert to normal.

In this paper, the conditions for determining alkaline phosphatase in the absence or presence of zinc were made to correspond as completely as possible to those of Roche. Two methods were employed: a) the Bodansky (6) method in which the phosphate split from B-glycerophosphate was measured, b) the Bessey, Lowry, and Brock (7) procedure based on the colorimetric determination of *p*-nitrophenol liberated from *p*-nitrophenyl-phosphate by the enzyme. The alkaline phosphatase in the presence and absence of zinc ion ($10^{-5} M$) was determined in a group of 23 normal healthy individuals and in 22 patients with advanced cancer.

With the aim of making the test more selective, a variety of conditions have been tested. These include the effect of varying the zinc ion concentration, of dialyzing the plasma, of changing the

order and manner of addition of the reagents and of altering periods of incubation.

Under Roche's conditions, the behavior of the cancer and non-cancer groups was found to be similar both qualitatively and quantitatively, i.e., inhibition of alkaline phosphatase by zinc ion.

EXPERIMENTAL

Fresh blood serum was taken from a number of healthy blood donors and from patients with far advanced cancer. Determinations for alkaline phosphatase were done according to Bodansky's method as employed by Roche. In several instances simultaneous analyses by the method of Lowry *et al.* were done. The results in which two concentrations of zinc sulfate and two periods of incubation were employed are listed in Tables 1 and 2.

Typical effects of zinc sulfate when added to dialyzed serum from a non-cancerous and a cancerous individual are indicated in Figure 1.

No significant change in the behavior of the test was found when the order of addition of the reagents was rearranged in every possible combination or when the determinations were performed at pH 8.9 instead of 9.2.

DISCUSSION

In agreement with Roche's data, zinc ion was found to cause a depression of the serum alkaline phosphatase in cancer patients. However, the identical behavior of the enzyme in the sera from non-cancer patients contrasts markedly with his findings. The failure of the serum alkaline phosphatase to be increased after dialysis is not consistent with Roche's view of an excess of circulating zinc ion originating from the tumor.

There is no obvious explanation which can adequately reconcile these two differing sets of data. It does not seem likely that a contaminant in the

* This work was supported by an institutional grant of the American Cancer Society, Inc., New York, and by funds from the National Institutes of Health, National Cancer Institute, Bethesda, Md.

TABLE 1

THE EFFECT OF ZINC ION ON THE SERUM ALKALINE PHOSPHATASE OF NON-CANCER PATIENTS

SUBJECT	UN-TREATED SERUM	ALKALINE PHOSPHATASE IN THE PRESENCE OF $ZnSO_4$			
		Concen- tration	Percent- age inhibi- tion	Concen- tration	Percent- age inhibi- tion
<i>Incubation time 1 hour</i>		$1 \times 10^{-5} M$			
M. H.	1.0	0.6			
F. M.	2.4	1.2	50		
M. D.	1.8	1.0	44		
R. H.	1.4	0.2	86		
R. C.	0.8	0.0	100		
R. C. D.	1.0	0.6	40		
A. R.	0.7	0.8	-(+14)		
A. J.	2.1	0.9	57		
<i>Incubation time 1 hour</i>		$4 \times 10^{-6} M$			
N. M.	3.3	2.2	33		
P. W.	2.9	2.1	27		
L. M.	3.9	2.6	33		
<i>Incubation time 3 hours</i>		$1 \times 10^{-5} M$			
H. S.	3.0	1.5	50	1.1	63
J. B.	2.3	1.2	48	0.7	70
M. W.	1.1	0.5	54	0.4	64
E. M.	1.1	0.5	54	0.4	64
H. C.	1.4	0.7	50	0.6	43
R. C.	2.2		0.8		63
W. W.	2.3	1.0	56	0.7	70
H. S.	1.3		0.4		69
J.	1.6	0.9	44	0.7	44
E. C.	1.2		0.4		67
T. K.	3.0		0.8		73
J. R.	2.7		0.5		82

reagents was responsible, because the inhibition of serum alkaline phosphatase by zinc ion in cancer patients was observed in both laboratories. Perhaps the continental population without cancer possesses an alkaline phosphatase system peculiarly sensitive to zinc ion. The necessity of varying zinc ion concentrations for establishing the optimum concentration of zinc ion activation in normal individuals has been pointed out by Roche (8). However, in our experience, this maneuver was unsuccessful in demonstrating activation.

Bodansky and Blumenfeld (9) found that "a concentration of $10^{-5} M Zn^{++}$ failed to affect the

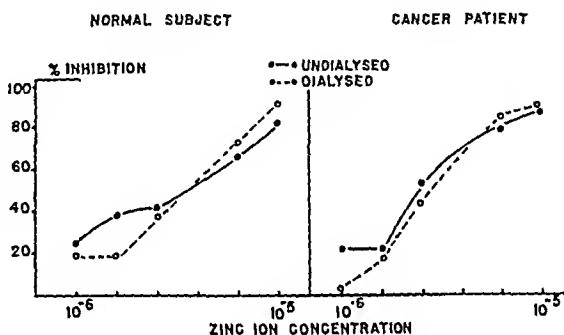


FIG. 1.—Effects of varying zinc ion concentrations on phosphatase activity (7) in dialysed and undialysed serum of patients with and without cancer.

TABLE 2

THE EFFECT OF ZINC ION ON THE SERUM ALKALINE PHOSPHATASE OF CANCER PATIENTS*

SUBJECT	DIAGNOSIS	ALKALINE PHOSPHATASE IN THE PRESENCE OF $ZnSO_4$				
		UNTREATED SERUM	Concen- tration $1 \times 10^{-5} M$	Percent- age inhibi- tion	Concen- tration $4 \times 10^{-6} M$	Percent- age inhibi- tion
<i>Incubation time 1 hour</i>						
B. K.	Renal cell carcinoma	2.6	1.4	46		
J. L.	Fibrosarcoma of cheek with pulmon. metastasis	4.6	2.0	57		
R. M.	Carcinoma, breast, with undifferentiated metastasis	6.6	2.3	65		
F. N.	Glioblastoma multiforme	2.0	0.5	75		
J. M.	Adenocarcinoma of rectum	2.1	0.7	66	1.4	33
E. D.	Adenocarcinoma of ovary	2.2	1.4	36	1.4	36
<i>Incubation time 3 hours</i>						
G.	Glioblastoma multiforme	2.2	0.7	68		
F. L.	Adenocarcinoma, urinary bladder	1.5	0.6	60	4.8	46
A. S.	Adenocarcinoma, breast	8.9	3.5	60		
P. C.	Epidermoid carcinoma	1.7	0.5	70		
N.	Squamous cell carcinoma of tongue, neck metastases	2.8	0.9	66	1.2	55
C.	Epidermoid carcinoma of tongue	2.7	0.9	67	1.2	55
S.	Scirrhous carcinoma of breast, spinal metastasis	2.1		0.5	76	
O.	Carcinoma of cecum	2.7		0.7	74	
D.	Carcinoma, prostate	21.6			17.0	21
T.	Carcinoma, breast	1.7			1.2	29
N.	Glioblastoma multiforme	1.9			0.6	68

* The same study was done with 5 other cancer patients not listed here and also on the patients whose data are found in Table 2, employing concentrations of zinc ion different from the two given. Essentially similar results were obtained.

serum alkaline phosphatase activity in individuals either with or without cancer." They also noted that "concentrations of 10^{-4} and $10^{-3} M$ decreased the serum alkaline phosphatase activity" (to the same extent in patients with and without malignancy). In their experiments, the pH of the digests was adjusted to 8.75 ± 0.05 because of the acidifying effect of $ZnSO_4$. In our work this was not done, in order to maintain experimental conditions as close as possible to those of Roche. The possibility exists that the observed inhibition may be the result of a shift of the pH towards the acid side rather than a direct effect of zinc ions.

Although in the future metallic ions may possibly be successfully employed to differentiate enzymatically patients with cancer from those without, the zinc ion inhibition of the serum alkaline phosphatase in its present state would appear to be unsuitable as a diagnostic test.

SUMMARY

Serum alkaline phosphatase from subjects with and without cancer was inhibited by zinc sulfate ($1 \times 10^{-5} M$) to the same extent. These data contrast with those of Roche in which the normal group showed no change or an activation of serum alkaline phosphatase by zinc ion.

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Influence of Thyroid Hormone on the Formation of Induced Skin Tumors in Mice*

HERBERT SILVERSTONE, PH.D., AND ALBERT TANNENBAUM, M.D.

(From the Department of Cancer Research, † Medical Research Institute, Michael Reese Hospital, Chicago 16, Illinois)

Chronic caloric restriction inhibits the formation of various tumors of the mouse (1, 2). The restriction in caloric intake produces, among many changes, a decreased total metabolism and decreased body weight of the animals. In order to acquire information as to which of these—caloric intake, total metabolism, or body weight—might be most directly related to tumor formation, diverse experimental procedures have been studied: feeding of dinitrophenol or sodium fluoride, and the housing of the mice at low environmental temperatures (3).

The present study supplements the above experiments and has a similar objective; in this instance thyroid hormone was fed to mice in a dose that caused increased food consumption and increased metabolism, and only a slight retardation of body growth. The experiments were designed to also reveal the effects of administration of thyroid on the different stages of carcinogenesis.

The concept of discrete stages in the genesis of tumors has been introduced through a variety of studies (4, 5, 6, 7, 8) indicating that the formation of induced skin tumors proceeds through at least 2 stages: a) initiatory changes induced by the action of a carcinogen on normal cells; and b) the promotion or development of these "biased" cells, under necessary conditions, into neoplastic cells that grow into a visible tumor. These two stages can be separated roughly by the experimental technique of applying a carcinogen for a short time and terminating these applications before tumors arise in any of the experimental animals. One may arbitrarily regard the consequent intervals—a) that encompassing the application of the carcinogen, and b) the subsequent period during which grossly visible tumors appear—as corresponding respectively to the stage of initiation and the stage of development. This technique has been employed to demonstrate that the inhibitory ac-

tion of caloric restriction (9) and the accelerating action of fat-enriched diets (10, 11) on the genesis of tumors are exerted mainly during the second stage, that of development, and have little or no effect on the initiation stage. Similarly the co-carcinogenic actions of croton oil (5), turpentine (4, 5), and wound healing (4) are effective mainly in the stage of development. On the other hand, various solvents for carcinogens influence tumor formation primarily in the initiatory stage by modifying the concentration or amount of carcinogen acting upon the tissue.

This general knowledge of the stages of carcinogenesis has been briefly reviewed, because the present study was in part based upon these considerations. The experiments were designed to study the effects on skin tumor formation of thyroid hormone when administered a) throughout the experiment, b) only during the interval of cutaneous application of carcinogen, and c) only during the period of tumor appearance.

METHODS

The mice employed were adult dba strain males bred in our laboratories. Litter mates were distributed between the several groups of each experiment so far as possible. From the time of weaning until transfer to the experimental diets they were fed a commercial ration, Purina fox chow checkers. In all experiments the animals were housed in groups of 5 in cages with solid bottoms. The carcinogen was a 0.3 per cent solution of 3,4-benzpyrene in acetone; at semi-weekly intervals a single drop (0.02 cc.) was applied to the interscapular area by means of a dropping pipet.

The experimental diets were composed of Purina fox chow meal, skimmed milk powder, and cornstarch, and were prepared as in earlier work (9). The thyroid extract (Proloid)¹ was incorpo-

* This investigation was supported (in part) by a research grant from the National Cancer Institute, U.S. Public Health Service, and by the Gustav E. Berlin Memorial Fund.

† Supported, in part, by the Michael Reese Research Foundation and the Foundation for Cancer Research, Chicago.

¹ Generously donated by The Maltine Company, N.Y. Proloid is a thyroglobulin equivalent to an equal weight of Armour's U.S.P. thyroid; 1 grain of Proloid corresponds to 169 micrograms of crystalline thyroxine in the thyroidectomized rat assay. It is also stated to have less toxic action than other thyroid preparations.

rated in the diets at the level of 0.04 per cent, inasmuch as previous experience had indicated that this concentration increased calorie intake by approximately 50 per cent, without a drastic effect on body weight. Where the design of the experiment called for changing the diet, this was done a few weeks after the final application of the carcinogen.

The general care of the animals, examination and evaluation of tumors and other pathology, and recording were carried out as previously described (9).

EXPERIMENTS

Experiment 1. Three month old dba males were distributed into 3 groups of 50 mice each. The diets were composed of Purina fox chow meal, 35 per cent; skimmed milk powder, 20 per cent; and cornstarch, 45 per cent. The ration at the level of 4 gms. daily per mouse was the control diet. The same ration at the level of 6 gms. and containing 0.04 per cent thyroid extract, was the thyroid diet. These amounts were approximately at the *ad libitum* level. Group h-CC² was fed the control diet, and groups h-TC, and h-TT were fed the thyroid diet. Three weeks after institution of the experimental diets, treatment with carcinogen was begun. Fourteen applications of the solution of benzpyrene were given at semi-weekly intervals over a period of 6 1/2 weeks; 3 weeks after the final application, the mice of group h-TC were transferred from the thyroid ration to the control ration; the rations of groups h-CC and h-TT were not changed. Thus group h-CC was fed the control diet and group h-TT the thyroid diet throughout the experiment, while group h-TC was given the thyroid diet for the period encompassing the application of carcinogen and the control diet during the period of tumor appearance.

Excluding the deaths caused by a laboratory accident during the twenty-fourth week of the experiment, very few of the mice without tumors died before the fortieth week. At this time the death rate in group h-TT suddenly increased—7 tumor-free mice as well as some with tumors died between the fortieth and forty-eighth week. For this reason the experiment was terminated.

During the course of the experiment the caloric intake was dependent on the ration fed. The mice on the control diet consumed, on the average, 3.9 to 4.0 gms. daily; those on the thyroid

² For convenience, the experimental groups are designated as follows: The initial letter designates the experiment. The first letter after the dash indicates the diet during the period of carcinogen administration. The second letter after the dash indicates the diet during the period of tumor appearance, again either C or T.

diet, 5.4 to 6.0 gms. In the same order, they drank approximately 3 cc. and 6 cc. of water daily. Although they consumed nearly 50 per cent more food, the mice on the thyroid diet weighed less than those on the control diet (Table 1a).

The formation of skin tumors is illustrated in Figure 1 and the data summarized in Table 1b. The results are discussed together with those of the following experiment.

Experiment 2. Two hundred dba male mice, approximately 2 1/2 months of age, were divided into 4 equivalent groups of 50 each. The ration consisted of Purina fox chow meal, 50 per cent;

TABLE 1a
GROWTH OF MICE IN EXPERIMENT 1

Group*	WEEKS AFTER INITIAL APPLICATION OF CARCINOGEN									
	-3	0	4	8	10	14	18	26	34	42
Mean body weight (grams)										
h-CC	26	30	33	35	36	37	38	37	37	36
h-TT	25	28	31	33	34	34	33	32	32	31
h-TC	25	28	30	32	32	33	33	33	33	34

* h-CC: control diet; h-TT: thyroid diet; h-TC: thyroid diet until 9½ weeks, control diet subsequently.

TABLE IV

EFFECT OF FEEDING THYROID HORMONE ON THE FORMATION OF INDUCED SKIN TUMORS (EXPERIMENT 1)

GROUP*	NUMBER OF MICE†	MICE WITH SKIN TUMORS		TIME OF APPEARANCE OF TUMORS‡ (WEEKS)		MICE TUMOR FREE AND ALIVE AT 48 WEEKS§	
		Mice per cent	Per cent	First	Mean		
h-CC	43	32	74	14	30.9 ± 1.6	9	
h-TT	39	27	69	10	26.2 ± 1.9	4	
h-TC	50	44	88	10	29.3 ± 1.4	6	

* See footnotes, Table Ia, for outline of dietary regimen.

† The total mice in each group was 50. The adjusted total is employed to calculate per cent tumors because of unequal death rates among the several groups (15).

[†] Time in weeks after initial application of carcinogen.

§ End of experiment.

skimmed milk powder, 25 per cent; cornstarch, 22.5 per cent; and brewers yeast,³ 2.5 per cent. This ration, compared with that of Experiment 1, contains a higher proportion of protein, mineral, and fat and a supplementary source of B-vitamins. The control diet consisted of 4.0 gms. of the ration daily, the thyroid diet of 6.0 gms. of the ration containing 0.04 per cent thyroid extract.

Four weeks after initiation of the experimental diets, the mice were given the first of 12 semi-weekly applications of the benzpyrene solution—over a period of 5 1/2 weeks. Three weeks after the final application of carcinogen the diets were either

³ Anheuser-Busch Strain K—generously donated by Anheuser-Busch Inc.

changed or continued according to the design of the experiment as tabulated:

Group	DIETARY REGIMENT DURING		(8½ weeks to end of experiment)
	Period of carcinogen application* (-4 to 8½ weeks)	Period of tumor appearance*	
n-CC†	control	control	
n-TT	thyroid	thyroid	
n-CT	control	thyroid	
n-TC	thyroid	control	

* Time is indicated in weeks after initial application of carcinogen.

† See footnote number 2.

The study was terminated 48 weeks after the initial application of carcinogen inasmuch as subsequent to this time there occurred a sharp increase in the death rate of the mice ingesting the thyroid diet. In the first 48 weeks of the experi-

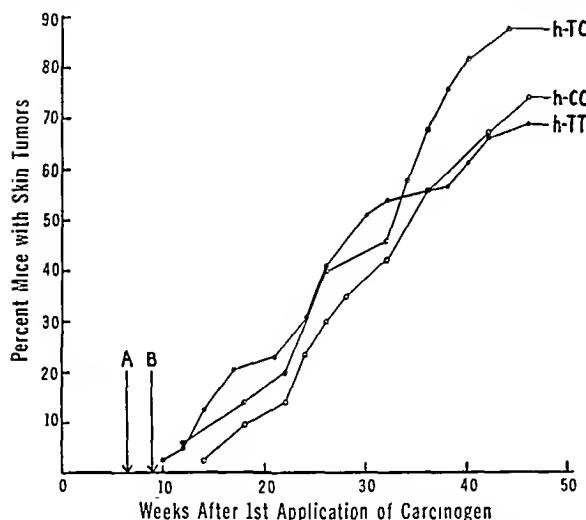


FIG. 1.—Effect of thyroid extract on the formation of induced skin tumors, as shown by curves of cumulative incidence. A: Time of final application of carcinogen. B: Group h-TC was fed thyroid diet until this time, the control diet subsequently. Group h-CC received the control diet throughout the experiment, and group h-TT received the thyroid diet throughout.

ment, there were only 2 to 5 deaths in a group, and most of these were caused by spontaneous lymphomata.

The mice receiving the control diet consumed from 3.7 to 4.0 gms. of food daily, those on the thyroid diet from 5.2 to 6.0 gms. The water consumption of the latter was from 25 to 50 per cent greater than that of the mice on the control ration. As in Experiment 1, the mice ingesting the thyroid diet weighed less than those on the control diet despite the increase in caloric consumption of approximately 40 per cent (Table 2a). On the average, the hyperthyroid mice were only about 2 per cent smaller in body length.

With respect to the formation of skin tumors,

the results of Experiment 2 (Table 2b, Figure 2) agree with those of Experiment 1 (Table 1b, Figure 1) in the following particulars: The initial tumors appeared earlier in those groups receiving the thyroid diets during the period of application of carcinogen (h-TT and h-TC compared with h-CC; and n-TT and n-TC compared with n-CC and n-CT). The tumor incidences among the mice fed the thyroid diet only during the period of carcinogen application, followed by the control diet during the period of tumor formation (groups

TABLE 2a
GROWTH OF MICE IN EXPERIMENT 2

Group*	WEEKS AFTER INITIAL APPLICATION OF CARCINOGEN									
	-4	1	5	9	11	15	19	27	35	43
Group*		Mean body weight (grams)								
n-CC	23	29	32	34	34	36	37	37	38	38
n-TT	22	28	31	32	32	32	33	33	34	32
n-CT	23	29	32	35	34	33	34	34	35	33
n-TC	23	29	31	32	31	34	37	38	38	37

* n-CC: control diet throughout; n-TT: thyroid diet throughout; n-CT: control diet until 8½ weeks, thyroid diet subsequently; n-TC: thyroid diet until 8½ weeks, control diet subsequently.

TABLE 2b
EFFECT OF FEEDING THYROID HORMONE DURING THE DIFFERENT STAGES OF CARCINOGENESIS ON THE FORMATION OF INDUCED SKIN TUMORS. (EXPERIMENT 2)

GROUP*	NUMBER OF MICE†	MICE WITH SKIN TUMORS	TIME OF APPEARANCE OF TUMORS‡ (WEEKS)		MICE ALIVE AT 48 WEEKS§	
			Num-ber	Per-cent		
n-CC	49	31	63	15	29.9±1.7	17
n-TT	49	25	51	11	28.0±1.9	21
n-CT	49	24	49	13	32.1±1.8	21
n-TC	49	34	69	9	28.4±1.6	12

* See footnote, Table 2a, for description of dietary regimen.

† Number of mice obtained by adjustments for deaths of animals without tumors (15).

‡ Time in weeks after initial application of carcinogen.

§ End of experiment.

h-TC and n-TC), were slightly greater than those of the mice on the control diets throughout the experiment. Tumor incidences among the mice fed the thyroid diet throughout (h-TT and n-TT) were at first slightly greater than among the control mice, but at about the thirtieth week there was a relatively sharp reduction of tumor formation among the hyperthyroid mice, and by the time the experiments were terminated these groups had slightly lower tumor incidences than the controls. As a consequence of this early augmentation and later repression of tumor formation the mean times of tumor appearance were shorter in groups h-TT and n-TT than in the corresponding control groups. The effect of feeding the thyroid diet only during the period of tumor appearance (i.e. preceded by the control diet during the period of ap-

pliation of carcinogen) is illustrated in Experiment 2, group n-CT. There was initially some inhibition of tumor formation (ending at about the twenty-seventh week) followed by an acceleration which raised the tumor incidence to that of the mice fed the thyroid diet throughout.

The values given for skin tumor formation in the tables and figures represent the total number and per cent of mice that developed skin tumors (papillomas and carcinomas). Approximately 70 per cent of the papillomas eventually became carcinomas, and the incidence of mice with carcinomas varied in the same order as given for total skin tumors.

DISCUSSION

The literature contains few reports on the effects of the administration of thyroid hormone on the genesis of neoplasms. Nowak and Ciechanowski (12) observed no change in the rate of appearance of tar-induced skin tumors in rabbits. On the other hand, Kreyberg (13) noted an acceleration of skin tumor formation in tarred mice. Smith and associates (14) reported no effect of the hyperthyroid state on the formation of induced sarcomas in rats; the result was attributed in part to the high dose of carcinogen employed. We have unpublished experiments in which mice injected with moderate doses of carcinogen were given thyroid extract in rations fed *ad libitum*; there were no noteworthy effects on sarcoma formation.

In the present experiments the striking augmentation of caloric intake and metabolic activity produced by the thyroid extract was not accompanied by a spectacular effect upon the incidence or time of appearance of skin tumors induced by the application of carcinogen. The data suggest, however, a small differential action of thyroid hormone during the two different stages of carcinogenesis. When fed during the period of application of carcinogen (initiation stage) it exerted a stimulating action on the formation of skin tumors, principally evidenced by the invariably earlier appearance of the first tumors in the four groups fed thyroid in this period, and by the higher incidence of tumors in the two groups fed thyroid extract during this period only. When administered during the period of tumor appearance (development stage), thyroid extract exerted some retarding effect on skin tumor formation, as indicated by the lower incidence of tumors. If these observations are valid, it would be expected that the feeding of thyroid extract throughout the entire experiment (both stages) would result in a balancing of the two effects. To a degree, the results are in agreement with this expectation.

The mechanisms of these actions are obscure.

The administration of thyroid hormone has many effects upon the animals: increased food consumption, increased metabolism, a relative loss in body weight, increased heat loss, peripheral dilatation of blood vessels, shifts and changes in protein and fat storage, etc. It is possible that the accelerating action on skin tumor formation during the initiation stage of carcinogenesis is due to local changes at the site and time of carcinogen application, possibly even affecting the tissue dose of carcinogen. Thus the augmented tumor formation reported by Kreyberg may have been due to protracted tar application (for 6 months), which contrasts with the relatively short period of car-

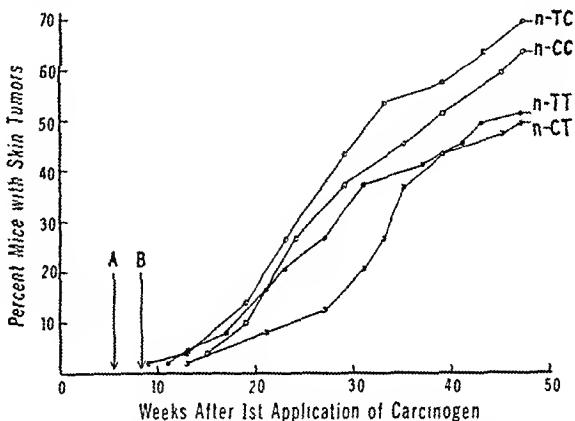


FIG. 2.—Effect of thyroid extract on the formation of induced skin tumors, as shown by curves of cumulative incidence. A: Time of final application of carcinogen. B: At this time group n-TC was transferred from thyroid to control diet, group n-CT from control to thyroid diet. Groups n-CC and n-TT were fed the control and thyroid diets respectively throughout the experiment.

cinogen application (for 5 1/2 and 6 1/2 weeks) in our experiments. It may be that the augmented tumor formation reported by him was due to a stimulatory action in the initiation stage of carcinogenesis which overlapped the period of tumor appearance and therefore outweighed the inhibitory effect during the development stage.

The small inhibiting effect of thyroid extract, on tumor formation, during the second stage of carcinogenesis may be related to the small decrease in body weight which occurred despite an increased food intake of 40 to 50 per cent. Thus the inhibition of tumor formation that occurred in the groups fed thyroid extract throughout the experiment or only during the second stage of carcinogenesis may be more directly related to the decrease in body weight rather than to the increased caloric intake and metabolic activity. These conclusions agree with the evidence that caloric restriction exerts its inhibiting effect

on tumor formation mainly during the second stage of carcinogenesis (9).

The most pertinent finding is that the considerable augmentation of food intake and metabolic activity, produced by thyroid extract, resulted in only a small effect upon tumor formation. These data support the conclusions arrived at in related investigations (3) concerned with dinitrophenol, sodium fluoride, and low environmental temperature: It is not the level of caloric intake or total metabolic turnover but rather the body weight level at which a balance is struck between caloric intake and utilization that is a significant factor in the genesis of some mouse tumors.

SUMMARY

1. Mice fed diets containing 0.04 per cent thyroid extract (Proloid) consumed 40 to 50 per cent more food, yet weighed about 10 per cent less, than the control mice.

2. The striking augmentation of calorie intake and metabolic activity did not produce a large effect upon the incidence or mean time of appearance of skin tumors induced by the application of 3,4-benzpyrene.

3. The data suggest, however, a small differential effect of thyroid hormone on the two stages of carcinogenesis: stimulating in the stage of initiation and retarding in the stage of development.

4. The results give indirect supporting evidence that the inhibition of tumor formation produced by caloric restriction is more related to the low body weight of the animal than to the actual level of caloric intake or metabolic activity.

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Vitamin B₆ and Biotin in Human Cancer Tissue*

R. M. BALLANTYNE AND E. W. McHENRY

(From the Department of Public Health Nutrition, University of Toronto, Toronto 5, Canada)

A great deal of experimental work has been done seeking some specific relation between the various vitamins and malignant neoplasms. The literature is extensive, but the diverse information is not readily fitted into a unified or consistent pattern for it is impossible to make extensive generalizations from the effects obtained with one type of tumor when the opposite effect may prevail with others. There are, however, three general approaches to the problem and with these in mind the scattered literature may be divided into first, the relation of vitamins to the induction of tumors; second, the relation of vitamins to the growth of tumors; and third, the vitamin content of tumors as compared with that of normal tissues. Our investigations fit into the third category but differ from most of the previous work in that we are dealing with spontaneous human cancers, not an experimental type induced or transplanted in animals.

As vitamin B₆ plays† an important part in the transamination process it was felt that the amount of this vitamin present in the neoplasm, compared with the amount present in the surrounding normal tissue, might serve as an indication of the amount of transamination taking place during neoplasia. In addition, the observation that the feeding of a diet low in pyridoxine caused the rats to be more resistant to the induction of cancer (9, 10) and also caused a reduction of growth of tumors already present (2, 8) prompted the study of the amount of vitamin B₆ present in various types of human neoplasms.

A few studies were made on the biotin content of neoplasms, for biotin also seems to be related to nitrogen metabolism as Winzler (12) has indicated in his work with yeast. Biotin has been reported to have a procarcinogenic effect on "butter yellow" induction of tumors (5, 7) and has been reported to affect the growth rate of tumors (3). In addi-

tion, the same assay method could be used, simplifying the experimental procedure.

EXPERIMENTAL

All human neoplasms were obtained through the cooperation of the Department of Surgical Pathology, Toronto General Hospital, and the Department of Pathology, Toronto Western Hospital. Sections of all tissues used for assay were made in these respective departments and microscopic examinations carried out. The diagnoses were received from these departments but no attempt was made to determine the density of stroma, the proportion of malignant to normal cells or the amount of necrosis present. All data represent the amounts of the vitamins present per gram of the entire tumor mass.

The tissue remaining after sections were made was available for assay and was prepared for determinations within six hours of removal in the case of surgical specimens and within six hours of death in the case of autopsy specimens. A preliminary experiment was carried out in which it was shown that there was no loss of vitamin B₆ during a six hour period following removal and thus the results represent the total amount of vitamin B₆ present in the tissue. Whenever possible vitamin estimations were carried out on surrounding normal tissue as well as on the neoplasm itself.

The vitamin B₆ and biotin content of the tumors was estimated microbiologically using the method of Atkin *et al.* (1) utilizing *Saccharomyces carlsbergensis*. For the assay of biotin, pyridoxine hydrochloride was substituted for biotin in the basal medium and a 0.3 milligamma/cc. solution of crystalline biotin was used as a standard. With this method the vitamin B₆ content of 40 malignant tumors and 20 samples of normal tissue was determined as well as the biotin content of 22 malignant tumors and 9 samples of normal tissue.

RESULTS

The vitamin B₆ content of the neoplasms fell within a range of 0.33 to 1.35 megm./gm., while the values for surrounding normal tissue fell within a much wider range from 0.26 to 8.90 megm./gm. A similar situation was found with the biotin

* This investigation was made possible by a grant from the Ontario Cancer Treatment and Research Foundation for which thanks are expressed.

† In this paper "vitamin B₆" is used to include pyridoxine, pyridoxal and pyridoxamine, in accordance with nomenclature approved by the American Institute of Nutrition on April 21, 1949.

values, where the range for the neoplasms was 0.02 to 0.16 megm./gm. Figures 1 and 2 depict the average values for the vitamin content of the various neoplasms and their host tissues.

DISCUSSION

An examination of these results indicates that a marked similarity of both vitamin B₆ and biotin content exists among all examined types of neoplasms, and that this similarity is not evident among the normal host tissues. Not only are the vitamin B₆ values within a narrow range but the

the greater is the departure from normal. Neoplasms are likely to be heterogeneous, being composed of both cellular and non-cellular elements in varying proportions and in addition having varying degrees of central necrosis which would effect the concentration values.

A chemical similarity has been previously demonstrated, the first indication being reported by Cori and Cori in 1925 (4) when they found that the laetic acid and sugar content of a sarcoma was very nearly the same as that of a carcinoma. Greenstein (6) has cited many instances where the nearly uniform metabolic pattern of neoplasms, of different etiology and histogenesis, have been reported. The similarity of the B vitamin content of neoplasms has been reported previously and our data, both quantitatively and with regard to uniformity in tumor tissue, are in general agreement with those found by Pollack, Taylor and Williams (11).

In several reports in the literature regarding the vitamin content of neoplasms the comparison between normal and neoplastic tissue has been made on a percentage basis. A determination of this sort

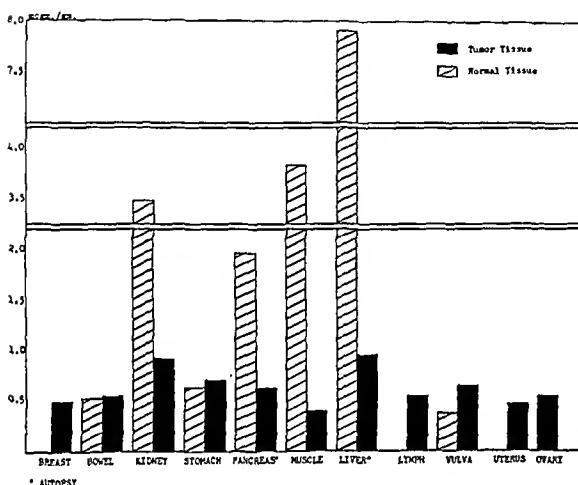


FIG. 1.—Vitamin B₆ content of human tissues

values are similar to those for normal tissues having a low vitamin B₆ content, such as the mucosa of the gastro-intestinal tract. Other tissues having high values for their vitamin B₆ content, such as liver, kidney and muscle, act as hosts for neoplasms which have small amounts of vitamin B₆. In fact the vitamin B₆ content of all malignant tumors studied was found to have a standard deviation of 0.19 megm./gm., whereas the corresponding group of normal tissues varied over such a wide range that the standard deviation could not be determined. A similar observation was made when the biotin content of tumors and of normal tissues was considered.

The standard deviation may be considered to be too high to be of significance, but there are not only a large number of different types of tumors, but these tumors, even those of the same type, may vary considerably in the degree to which they resemble or depart from the characteristics of normal tissue. In human neoplasms particularly, there are elaborate gradations of malignancy and it has been said that the greater the degree of malignan-

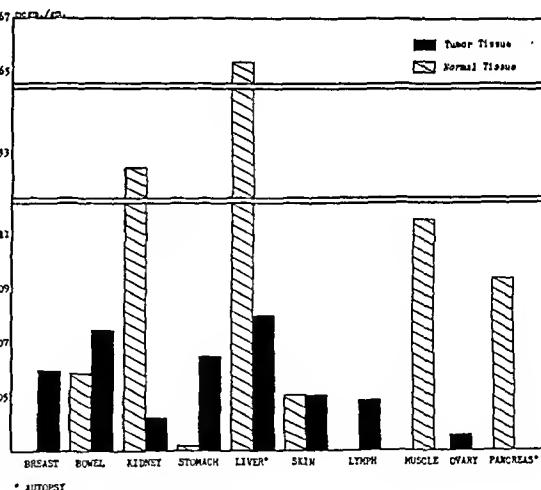


FIG. 2.—Biotin content of human tissues

can be misleading as the foregoing results show no relationship between the neoplasms and their tissues of origin. For example, if the vitamin B₆ content of bowel mucosa were compared with that of normal bowel mucosa, it would be regarded as 102 per cent of normal, whereas if the value for liver carcinoma were compared with that of normal liver, it would be 11 per cent of normal. These are extreme examples but indicate the wide difference and the lack of quantitative relationship between normal and tumor tissues.

SUMMARY

The vitamin B₆ content of 40 human malignant tumors and 20 samples of human normal tissue was determined, as well as the biotin content of 22 malignant tumors and 9 samples of human normal tissue. The vitamin B₆ content of all malignant tumors, irrespective of origin, fell within a narrow range of 0.33 to 1.35 megn./gm. This range was similar to that of those normal tissues having a low vitamin B₆ content. The various normal tissues studied had a wide range of values, from 0.26 to 8.90 megn./gm. The biotin content of all malignant tumors fell within the range of 0.02 to 0.16 megn./gm., while the normal tissues had a range of 0.03 to 0.66 megn./gm. The vitamin content of the neoplasms did not seem to bear any relation to the vitamin content of the surrounding normal tissue.

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Histopathologic Study of the Mode of Inhibition of Cellular Proliferation: Effect of 4-Dimethylaminostilbene on the Growth of Walker Rat Carcinoma 256*

JOHN W. GREEN, JR., M.D., AND C. C. LUSHBAUGH, M.D.

(From the Department of Pathology, The University of Chicago, Chicago 37, Illinois)

INTRODUCTION

Many classes of chemical compounds have recently been described which inhibit the growth of experimental animal tumors. Some of these compounds such as nitrogen mustard and urethane have been used in the chemotherapy of human lymphomas (1, 2, 3, 13, 16) and inoperable cancer (1, 8, 12). Recent studies with urethane in the treatment of experimental tumors have suggested that the mode of action of this chemotherapeutic agent may be due to its non-specific effect on debilitation of the host, together with some more specific action as the production of "fixed post-mitotic" cells (5, 9). If the mode of action of various classes of tumor inhibiting drugs can be demonstrated, then the common denominators of the different types of inhibitory chemicals may be found, and more active and less toxic inhibitory drugs synthesized.

The present study was undertaken to determine the mode of action of a new class of compounds, the aminostilbenes, described by Haddow and his group (11). They have shown that several of these compounds produce marked inhibition of growth of the Walker rat carcinoma 256 (11), that the degree of tumor inhibition is related to the dietary protein intake of the hosts (6, 11), and that they are powerful carcinogens (11).

This type of compound was selected to study for its mode of action because it is the prototype of water insoluble organic carcinogens, and its mode of action can be compared with the water soluble carcinogen, urethane, which has recently been extensively studied (9, 10).

MATERIALS AND METHODS

Two hundred and one male and 20 female albino rats, obtained mostly from Sprague-Dawley Farms, were used in these experiments. Their weights ranged from 200 to 350 grams, averaging 250 grams. The stock

animals were maintained on a diet of laboratory chow.¹ After preliminary experiments in which the animals were fed chow, synthetic diets were used in all subsequent experiments to facilitate paired feeding of the controls. The synthetic diet most frequently used contained 22 per cent casein.² In order to provide a low protein diet in some experiments a 5 per cent casein diet was used³ and in some a casein free 2 per cent carrot protein diet was used.⁴ In every experiment in which synthetic diets were used the controls were pair fed to the dietary intake of the aminostilbene treated animals. Dietary consumption was measured daily and the animals were weighed every other day. Because the Walker tumor has been extensively used in the study of the chemotherapy of transplantable rat tumors, this tumor was used in all the following experiments. The details of transplantation of the tumor have been previously reported (9). A dilute sterile saline suspension of tumor was inoculated subcutaneously over the spine. By the fourth day a measurable nodule of tumor had developed. Beginning on this day measurements of the length, width, and height of the tumors were made with a caliper every other day for the next 10 days. The resulting estimates of tumor volume were computed and their logarithms plotted against the logarithm of the day of growth. The growth curves of various experiments could then be compared as they were straight lines. At 14 days the animals were sacrificed, and the tumors excised and weighed. The mean weight of control tumors divided by the mean weight of treated tumors formed the ratio of inhibition for a given experiment. The significance of the difference of the mean tumor weights in

¹ Purina Laboratory Chow.

² Casein carrot diet, composition per 100 gm.: casein 22 gm.; carrots 30 gm.; ruffex 5 gm.; lard 4 gm.; cornstarch 22 gm.; salt mixture 4 gm.; liver concentrate 1 gm.; brewer's yeast 2 gm.; water 10 ml.; riboflavin 500 microgm.; pyridoxine hydrochloride 200 microgm.; calcium pantothenate 200 microgm.; choline chloride 100 mg.; vitamin A 200 USP units; and vitamin D 29 USP units (20).

³ Five per cent casein diet identical in composition and isocaloric with the 22 per cent casein diet, except that it contains 5 gm. casein and 39 gm. cornstarch per 100 gm. diet.

⁴ Casein free 2 per cent carrot protein diet isocaloric with the previous diets, and identical in composition except that the casein is replaced by cornstarch so that it contains 44 gm. cornstarch per 100 gm. diet (20).

* This study was aided in part by a grant from the Committee on Growth acting for the American Cancer Society.

control and treated groups was analyzed and only those experiments with a *p* value of 0.01 or less accepted for study.

Sections of the tumors fixed in formalin, embedded and sectioned in paraffin, were stained with hematoxylin and eosin, Wilder's silver reticulum (19), and Giemsa stains. In some experiments acetate-orecin crush preparations were made of the tumors to facilitate detailed study of mitotic figures. In addition to the usual methods of histologic study, some groups of tumors were evaluated quantitatively by the method of Chalkley (4) for the presence of tumor cells, tumor nuclei, fibrous tissue, and tumor cells in mitosis. The Chalkley method is based on the random distribution of four points in the microscope objective falling in the field of a section. It was determined that reproducible results could be obtained with this method by counting a minimum of 200

After the third injection diethylstilbestrol in oil was given subcutaneously to both groups of animals, 10 micrograms per rat. Pairs of rats in each group were sacrificed at 24, 48, and 72 hours after the single injection of stilbestrol. Seven and a half hours before each pair was sacrificed they were given colchicine intraperitoneally. When the animals were sacrificed the vagina and uterus were removed, bisected longitudinally, embedded in paraffin and cut and stained with hematoxylin and eosin. Mitoses were counted in 1000 cells in a strip 5 cells wide along the basal vaginal epithelial layer, and the mitotic rate for this tissue calculated.

EXPERIMENTS AND RESULTS

The Effect of 4-dimethylaminostilbene Hydrochloride on the Growth of the Tumor.—In the first

TABLE I
RELATIONSHIP BETWEEN THE DIET, TUMOR WEIGHT, AND BODY WEIGHT IN 4-DIMETHYLAMINOSTILBENE HYDROCHLORIDE TREATED AND UNTREATED RATS.

Experiment	No. of rats	Diel*	Av. wt. on day 1	Av. wt. on day 14	Change net wt.	Av. wt. (gm.)	Ratio of tumor inhibition	Mitotic rate per 400 cells	ANALYSIS OF TISSUE COMPONENTS		
									Percent nuclei	Percent cytoplasm	Percent fibrous tissue
I	10	Chow	244.8	210.66	-34.52	0.38	31.0	7.0	85.60	5.75	12.40
	10		252.6	258.0	-6.33	11.73			20.40	75.00	1.80
II	10	Chow	272.0	250.66	-23.98	2.68	5.70	7.90	83.60	8.11	11.60
	10		268.8	277.8	-5.74	14.74			20.50	75.80	1.90
III	10	Starve	299.3	244.33	-75.8	0.86	18.70	0.25	7.50	85.00	7.20
	10		296.6	233.3	-79.33	16.03			21.60	74.60	2.20
IV	7	22%	239.4	225.8	-20.15	0.55	3.68	3.50	5.75	11.30	
	7	casein	240.4	264.14	-0.37	24.11					
	7	5%	235.0	195.14	-43.32	3.18					
	7	casein	234.7	292.1	-34.81	22.21					

* See footnotes 2 and 3.

hits per slide in the analysis for various tissue components and 400 hits per slide in the determination of mitotic frequency.

The stilbene compound used in these experiments was 4-dimethylaminostilbene hydrochloride, M.P. 174-175, synthesized by the method of Saehs (17).⁵ It was dissolved in propylene glycol at a concentration of 100 mg. per ml., and injected intraperitoneally at a dose of 50 mg. per kilogram of body weight. Injections were begun on the fourth day of tumor growth, and continued every other day for a total of 5 injections. Control animals were similarly given 0.5 ml. propylene glycol per kilogram at the same time intervals.

Colchicine was used to arrest mitosis in several experiments. It was administered in doses of 1 mg. per kilogram intraperitoneally 7.5 hours before the animals were sacrificed.

The female rats were ovariectomized and allowed to recover from the operation. They were not inoculated with the tumor. After being divided into two groups, one group was given the stilbene compound for three injections, while the controls were given propylene glycol.

⁵ Synthesized by Mrs. Z. Tillitson, of the University of Chicago Toxicity Laboratory.

experiment the growth of the tumor was studied in 20 rats fed dog chow *ad libitum*. The treated tumors averaged 0.38 grams in weight when excised on the fourteenth day, while the control tumors averaged 11.73 grams, giving a ratio of inhibition of 31.0. The net weight change averaged -34.52 grams in the treated animals and -6.3 grams in the control tumor bearing animals. This experiment was repeated with a resultant ratio of inhibition of 5.7 (Table I). In the second experiment the weight change for the treated and control animals was -23.98 and -5.74 grams respectively. Since the ratio of inhibition varied with the weight loss of the treated animals, in the third experiment, beginning on the fourth day both groups were starved. The net weight changes in this experiment were -75.8 grams in the treated and -79.33 grams in the controls. The ratio of inhibition was 18.70. The growth curves for both groups of animals are shown in Fig. 1.

Since it has been shown that the protein content of the diet has a direct effect on the degree of

tumor inhibition produced by the stilbene compounds (6, 11), a series of experiments was done in which a high and a low protein diet was used. Four groups of 7 animals each were used; two groups were on the 22 per cent casein diet and 2 groups were on the 5 per cent casein diet. In each dietary group the controls were pair fed to the consumption of the treated groups. Net weight changes and tumor weights are shown in Table 1, Experiment IV. There was a consistently greater loss of weight in the treated animals as compared with the con-

tumors. All of the tumors in these experiments were fixed in acetic-orcein and crush preparations for mitotic figures were made.

The growth curve for this experiment is compared with those of other representative experiments in Fig. 1. This experiment was repeated using 42 animals and adding a casein free 2 per cent carrot protein diet. Inhibition of tumor growth of the same order as in previous experiments occurred. On the eighth and twelfth days of tumor growth, half of each dietary group was given colchicine and sacrificed 7.5 hours later for mitotic counts. When treatment of a group of animals was stopped and they were allowed to live, the tumors resumed growth in 2 to 4 days. The animals then either died from necrosis and sepsis of their tumors or from massive hemorrhage from acute gastric ulceration. The gastric lesion has also been described by Haddow (11).

Histologic Studies of the Effect of 4-dimethylaminostilbene Hydrochloride on the Morphology of the Walker Tumor.—Microscopic study of the 14 day old treated tumors revealed a striking change from the control histology. This change was seen particularly in those tumors whose hosts had been starved, or fed low protein diets. A similar picture was seen in the tumors of the treated chow fed hosts; since their dietary intake was so poor, they were actually on a low protein diet. The treated tumors showed a replacement of the usually homogeneous tumor cells by pleomorphic giant cells. Occasional multinucleated giant cells were observed (Figures 4 and 5). The tumor giant cells were never observed in mitosis. In addition there was a heavy overgrowth of fibrous stroma and reticulum (Figures 8 and 9). The histologic changes in those treated tumors whose hosts were fed the high protein synthetic diet were not as marked; this group also showed the least weight loss. However, the morphology of the tumors of these animals was definitely changed from that of the controls (Figures 2 and 3). The reticulum showed a marked increase over that seen in the control tumors and there was some increase in tumor cell size (Figures 6 and 7). There were few tumor giant cells seen.

The results of analysis of the histological components of some tumors by the Chalkley method are shown in Table 1. There was a great increase in fibrous tissue and a decrease in hits on tumor cell nuclei. The hits on the tumor cell cytoplasm were increased. A comparison of the mitotic rate, net weight change of the host, tumor weight and dietary regimen is shown in Table 1. On both the 22 per cent and 5 per cent casein diets the mitotic rate for the treated animals was slightly less than

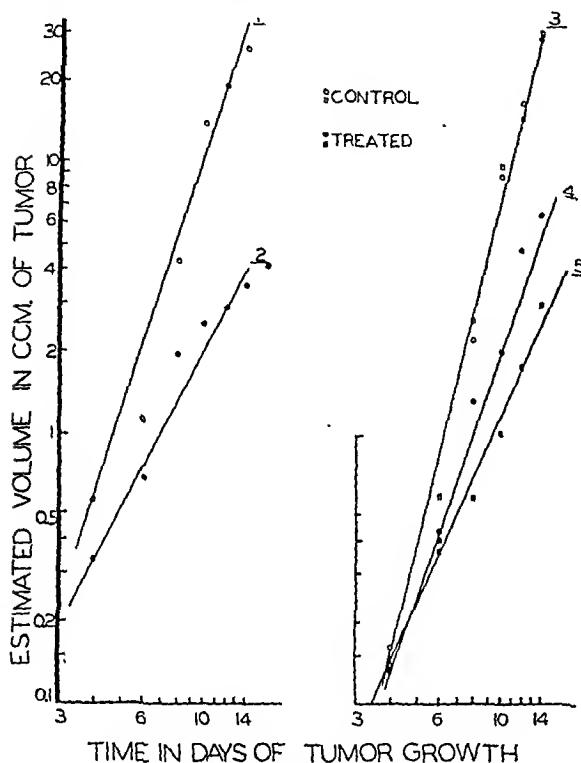
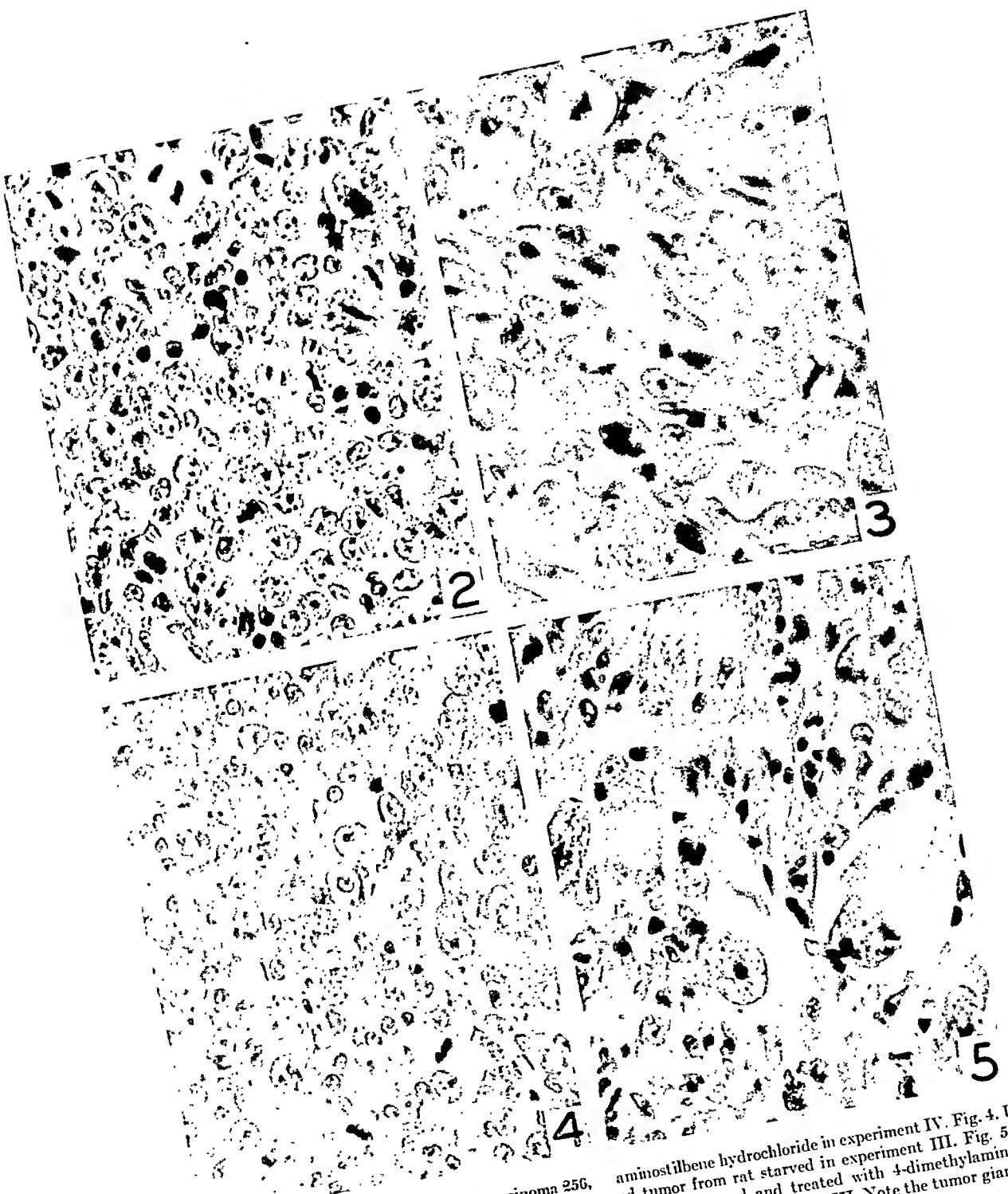


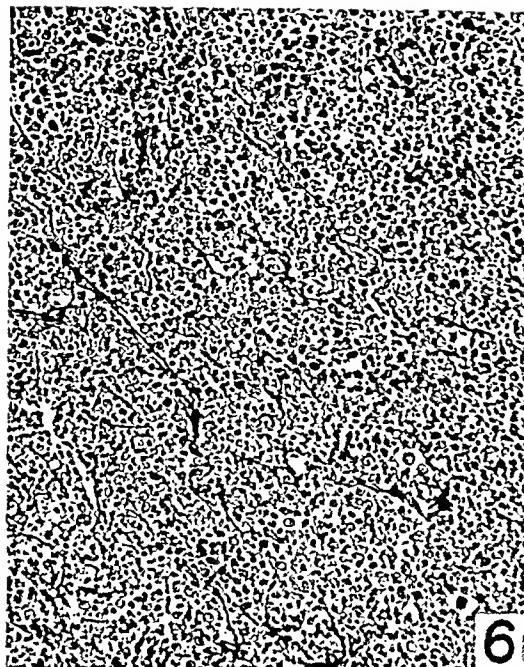
FIG. 1.—The growth rates of Walker carcinoma 256 in rats fed various diets, with and without treatment with 4-dimethylaminostilbene hydrochloride, plotted on a log-log scale. The points indicate the estimated tumor volumes in the following dietary groups: 1, control rats fed laboratory chow; 2, treated rats fed the same diet; 3, control rats fed 22 per cent casein diet (open circles) and control rats fed 5 per cent casein diet (open squares); 4, treated rats fed 22 per cent casein diet; 5, treated rats fed 5 per cent casein diet. The control animals represented by 3 were restricted to the same daily dietary intake as their corresponding treated groups, 4 and 5.

trols, even though the dietary intake of both was the same. The treated tumors of the animals on the 22 per cent casein diet averaged 6.55 grams in weight, those on the 5 per cent protein diet, 3.18 grams. The control tumors averaged 24.11 grams in the high protein, and 22.21 grams in the low protein groups. The 5 per cent protein diet did not significantly decrease the weight of the control

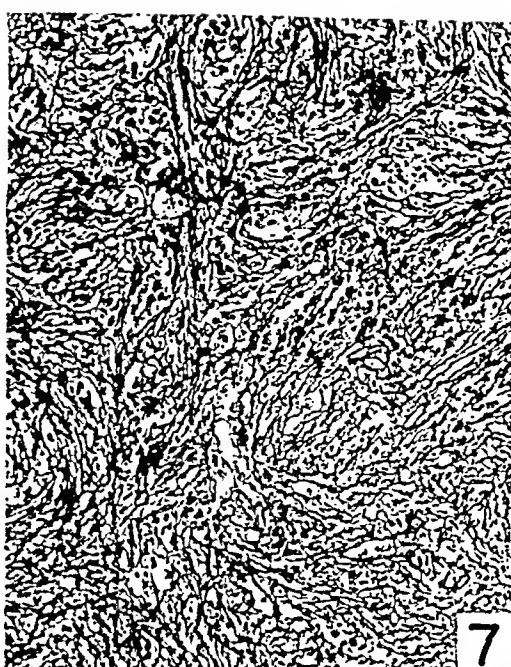


Figs. 2 to 5.—Photomicrographs of Walker carcinoma 256, 14 days old. Hematoxylin and eosin stains. $\times 570$. Fig. 2. Untreated tumor from rat pair-fed 22 per cent casein diet in experiment IV. Fig. 3. Tumor from rat treated with 4-dimethyl-

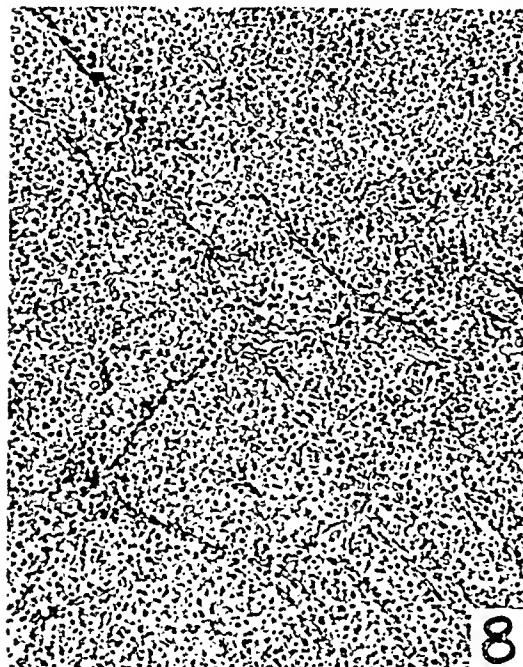
aminostilbene hydrochloride in experiment IV. Fig. 4. Untreated tumor from rat starved in experiment III. Fig. 5. Tumor from rat starved and treated with 4-dimethylaminostilbene hydrochloride in experiment III. Note the tumor giant cells.



6



7



8



9

Figs. 6 to 9.—Photomicrographs of the same tumors shown in Figs. 2 to 5, stained with Wilder's technique for silver im-

pregnation of reticulum. $\times 125$. Fig. 6. Same as Fig. 2. Fig. 7. Same as Fig. 3. Fig. 8. Same as Fig. 4. Fig. 9, Same as Fig. 5.

the controls. That this was not due to more severe nutritional debilitation of the treated animals is shown by Experiment III (Table 1) where both treated and control groups were starved. Although the net weight loss of the controls was 79.33 grams, and that of the treated 75.8 grams, the mitotic rate was 4.5 and 0.25 per 400 cells respectively.

The small consistent difference between the treated and control tumors was greatly accentuated by the administration of colchicine on the eighth and twelfth days of tumor growth. After colchicine the control tumors, regardless of the type of host diet, showed a consistently higher rate of mitosis (Table 2). While this marked inhibition of tumor

er and more sustained increase. This inhibition of mitosis in the treated animals was not the result of severe weight loss (Table 3). Controls restricted in food intake to the weight loss of the treated animals still showed a good estrogenic response and a marked increase in the mitotic rate of the vaginal basal epithelium. The mitotic figures of both treated and untreated tumors were studied to determine if there was an increase in abnormal mitotic figures which might give rise to post mitotic cells that could not contribute any further increase in tumor size such as occurs in urethane treated Walker tumors (9). The animals on which these studies were made were pair fed a 22 per cent

TABLE 2

EFFECT OF COLCHICINE ON THE NUMBER OF MITOSIS IN WALKER RAT TUMOR 256 AND THE JEJUNAL CRYPTS OF RATS AFTER TREATMENT WITH 4-DIMETHYLAMINOSTILBENE HYDROCHLORIDE.*†

Treatment	Diet	TUMOR				JEJUNUM			
		Number mitoses per 400 cells.		Number pyknotic nuclei per 400 cells.		Number mitoses per 200 crypt cells.		Number pyknotic nuclei per 200 crypt cells.	
		8 Days	12 Days	8 Days	12 Days	8 Days	12 Days	8 Days	12 Days
Control	22% casein	10.0	12.0	0.0	0.0	3.5	7.5	0.0	0.0
Colchicine	"	37.0	61.5	3.0	0.5	28.5	48.0	37.0	46.0
Aminostilbene	"	4.0	3.0	0.0	0.0	4.0	3.0	0.0	0.0
Aminostilbene and colchicine	"	11.0	0.0	6.5	0.0	25.0	52.0	42.5	31.0
Control	5% casein	14.5	7.0	0.0	0.5	4.5	4.5	0.0	0.0
Colchicine	"	54.5	32.0	1.5	8.5	44.0	44.0	47.0	23.0
Aminostilbene	"	4.5	4.0	0.5	1.0	2.0	5.0	0.0	0.5
Aminostilbene and colchicine	"	8.0	5.5	4.5	0.5	36.5	41.0	56.0	25.0
Control	2% carrot protein	9.0	6.0	0.0	0.0	4.0	4.0	0.5	0.0
Colchicine	"	80.5	53.5	3.5	7.0	23.0	31.5	59.5	54.5
Aminostilbene	"	0.5	2.5	0.0	0.0	1.0	4.5	1.0	0.0
Aminostilbene and colchicine	"	5.5	3.0	1.0	0.5	59.5	36.5	36.5	22.0

* The 8 day treated animals received 3 injections of the stilbene compound, the 12 day animals 5 injections.

† Four rats used in each group.

cell mitosis by 4-dimethylaminostilbene hydrochloride was observed, the mitotic rate of the jejunal crypts remained essentially unchanged in both treated and control animals (Table 2).

The observation that this was an apparent specific inhibition of tumor cell division by the stilbene compound while the intestinal crypts remained unaffected suggested the study of another rapidly proliferating normal tissue. The vaginal epithelium of the ovariectomized rat under the influence of estrogen was used for this purpose (18). After the third injection of the stilbene compound and the administration of stilbestrol, it was observed that estrus was suppressed in the treated animals. While the mitotic rate of the vaginal epithelium of the treated animals showed some slight increase, the control animals showed a much greater

increase and a 5 per cent casein diet. No colchicine was used in these experiments. All tumors used in the mitotic study were 14 days old. The abnormal mitoses were considered to be (9): polypoid prophase, cells with greatly increased numbers of scattered chromosomes; abnormal metaphase, poor spindle formation due to chromosome clumping and omission of chromosomes from the spindle; sticking chromosomes, chromosomes remaining attached after metaphase and failing to enter into daughter cells in anaphase; lagging anaphase, the lagging-behind of one or more chromosomes as the daughter cells are formed; bizarre figures, multiple spindles in a single cell or any peculiar configurations that could not be readily classified. Table 4 shows the results of this detailed study of mitotic figures. The difference in dietary protein had no

TABLE 3

THE EFFECT OF 4-DIMETHYLAMINOSTILBENE HYDROCHLORIDE ON THE VAGINAL EPITHELIUM OF THE OVARIECTOMIZED FEMALE RAT, AT 24 HOUR INTERVALS AFTER THE ADMINISTRATION OF DIETHYLSILBESTROL, 10 MICROGRAMS PER RAT. (COLCHICINE 1 MG/KG GIVEN $\frac{1}{2}$ HOURS BEFORE EACH GROUP SACRIFICED)* 1000 CELLS COUNTED IN EACH RAT.

Animal No.	Treatment	Time after stilbestrol	Weight change (grams)	Mitoses per 1000 cells
1	Treated	24 Hours	-50	36
2	"	24 Hours	-50	32
3	Control	24 Hours	-3	66
4	"	24 Hours	+3	68
5	"	24 Hours	-53	66
6	Treated	48 Hours	-23	46
7	"	48 Hours	-33	33
8	Control	48 Hours	-6	173
9	"	48 Hours	-16	266
10	"	48 Hours	-60	111
11	"	48 Hours	-55	327
12	Treated	72 Hours	-60	66
13	"	72 Hours	+17	38
14	Control	72 Hours	-25	67
15	"	72 Hours	-22	98
16	"	72 Hours	-58	149
17	"	72 Hours	-56	171

* The 24 hour treated animals were given 3 injections of the stilbene compound; the 48 hour and 72 hour animals were given 4 injections.

effect on the incidence of abnormal figures. In the control tumors of both dietary groups about 30 per cent of the figures were considered to be abnormal. The greatest increase in abnormal appearing mitotic figures in the treated tumors was in abnormal metaphases.

COMMENT

It would seem from these experiments that 4-dimethylaminostilbene inhibits the growth of the Walker rat carcinoma 256 primarily by direct mechanisms. Even though both treated and untreated animals were depleted of the same amounts of weight in Experiment III (75.8 and 79.33 grams, respectively), the marked difference in mitotic rate of the two groups shows that non-specific nutritional debilitation is not a primary factor in the restriction of tumor growth. That specific debilitation plays some role in tumor inhibition with this compound has been shown by Elson and Haddow (6) and confirmed in the experiments described above. Although a 5 per cent casein diet had no effect on control tumor growth in these experiments, the low protein diet increased the ratio of inhibition from 3.68 to 6.98. The degree of tumor inhibition

TABLE 4
ANALYSIS OF MITOTIC FIGURES OF 14 DAY OLD CONTROL AND 4-DIMETHYLAMINOSTILBENE HYDROCHLORIDE TREATED WALKER TUMORS.*

Animal No.	Diet	NORMAL MITOTIC FIGURES					ABNORMAL MITOTIC FIGURES						Total abnor- mal (per cent)
		Pro- phase	Meta- phase	Ana- phase	Telo- phase	Degener- ing cells	Total normal (per cent)	Poly- ploid pro- phase	Abnor- mal meta- phase	Stick- ing chromo- somes	Lag- ging ana- phase	Bi- zarre figures	
Control:	22% casein												
1	casein	65	1	0	0	8	74	0	10	8	7	1	26
2	"	53	9	0	0	2	64	0	23	2	11	0	36
3	"	62	3	0	0	1	66	3	17	5	9	0	34
4	"	61	7	0	0	6	74	2	16	6	2	0	26
5	"	72	4	0	0	2	78	0	9	2	11	0	22
6	"	49	10	0	1	7	67	1	10	4	17	1	33
AVERAGES		60.3	5.6	0	.17	4.3	70.5	1	14.1	4.5	9.5	.3	29.5
Treated:	22% casein												
1	casein	23	3	0	1	4	31	6	45	3	15	0	69
2	"	26	4	0	1	6	37	1	30	7	25	0	63
3	"	15	3	0	0	4	22	4	47	7	14	6	78
4	"	47	1	0	0	8	56	4	30	3	7	0	44
5	"	22	2	0	0	4	28	6	42	10	14	0	72
6	"	15	3	0	1	9	28	3	51	2	16	0	72
AVERAGES		24.6	2.6	0	.5	5.8	33.6	4	40.8	5.3	15.1	1	66.3
Control:	5% casein												
1	casein	51	9	1	13	11	85	3	2	2	8	0	15
2	"	57	9	0	3	10	79	2	9	4	6	0	21
3	"	49	13	2	2	7	73	0	11	5	11	0	27
4	"	51	4	0	0	2	57	0	26	1	15	1	43
5	"	38	10	0	1	9	58	0	22	4	15	1	42
AVERAGES		49.2	9.0	.6	3.8	7.8	70.4	1	14	3.2	11	.4	29.6
Treated:	5% casein												
1	casein	47	0	0	0	2	49	8	37	1	5	0	51
2	"	29	1	0	0	6	36	9	45	0	8	2	64
3	"	41	4	0	0	7	52	9	16	13	9	1	48
4	"	38	3	0	1	3	45	1	41	1	10	2	55
5	"	36	2	0	1	1	40	2	44	0	8	6	60
AVERAGES		38.2	2	0	.4	3.8	44.4	5.8	36.6	3.0	8	2.2	55.6

* One hundred mitoses were studied in each tumor.

tion must be correlated in some manner with protein synthesis. Recent work by Feinstein (7) suggests more specifically that one effect of 4-dimethylaminostilbene on protein metabolism is a marked reduction in liver and splenic cathepsin rate, which might slow the normal rate of protein turnover. This could lead to a diminution in available protein for tumor and body growth.

The effect of inhibition of mitosis by 4-dimethylaminostilbene hydrochloride is independent of the dietary intake, and occurs to about the same extent in tumor bearing animals fed a 22 per cent or 5 per cent casein, or 2 per cent carrot protein diet for short periods of time. This would suggest that the ability to inhibit mitosis is an independent factor not related directly to dietary intake. The observation that the mitotic rate of the jejunal crypts was not affected while that of the tumor was decreased implies a very specific mitotic inhibitory ability of the drug. However, since it will also inhibit the mitosis of the vaginal epithelium of ovariectomized rats when stilbestrol is administered, it is not simply a specific inhibitor of neoplastic cells.

The ability of 4-dimethylaminostilbene to produce an increase in abnormal mitotic figures is similar to the observations made on human tumors treated with x-ray (14, 15), and on urethane treated rat tumors (9). In the latter study it was suggested that the abnormal mitotic figures might give rise to cells which were incapable of further division, and hence post mitotic cells. These post mitotic cells could not add any subsequent generations of cells to the tumor mass. This mechanism is also apparently a factor in the inhibition of 4-dimethylaminostilbene treated tumors.

The striking change in the histological appearance of the treated tumors may be the result of these mechanisms. The slower growth of the tumor gives the stroma of the host more opportunity to supply a dense matrix than in a very rapidly growing tumor. The peculiar atypical cells in the treated tumors (Figure 5) may arise from abnormal mitotic figures and represent the final stage of the fixed post mitotic cell. That this is possible is suggested from the observation that none of these cells have ever been observed in mitosis.

While the mechanism of tumor inhibition by urethane is apparently due to the non-specific action of the debilitating effect of the drug combined with the specific effect of the production of abnormal mitotic figures giving rise to post mitotic tumor cells, the inhibitory effect of 4-dimethylaminostilbene is more specific. The debilitation by reduction in dietary protein intake increases the effect of this drug on tumor growth. The produc-

tion of abnormal mitotic figures and the inhibitory effect of tumor cell mitosis is apparently independent of nutritional debilitation.

SUMMARY

The inhibitory effects of 4-dimethylaminostilbene hydrochloride on cellular proliferation have been studied in the Walker rat carcinoma 256, jejunal crypts of intact male, and vaginal basal epithelium of the ovariectomized rat. The findings of Haddow and his group have been confirmed (6, 11), that this compound is a powerful inhibitor of tumors and its inhibitory effect is dependent in part on the nutritional state of the host. It has been shown that a spectacular change in the histologic appearance of the tumors occurs under the influence of this drug, and that this histologic change of tumor giant cell formation, overgrowth of fibroblasts, and heavy reticulum formation is greatest in those tumors whose hosts have had the poorest nutritional state and lost the most weight. In addition it has been found that in tumors treated with aminostilbene there is an increase in incidence of abnormal mitotic figures of the type which are postulated to give rise to post mitotic cells. It is suggested that these post mitotic cells may not be available for further tumor growth. The more specific effect of inhibition of mitosis of tumor cells by this drug has been described. This inhibition of mitosis has been found whether the host was on either a high or low protein diet. The jejunal crypt mitotic rate was not affected by the drug in these animals. This compound was also found to inhibit the vaginal epithelium of the ovariectomized rat which had been given stilbestrol. The inhibition of mitosis of the stilbestrol stimulated vaginal epithelium of the rat is not due to marked weight loss of the treated animals.

On the basis of this study 4-dimethylaminostilbene hydrochloride appears to inhibit tumor growth by: production of abnormal mitotic figures giving rise to post mitotic cells, inhibition of tumor cell mitosis, and by interfering with normal protein synthesis in the tumor bearing rat.

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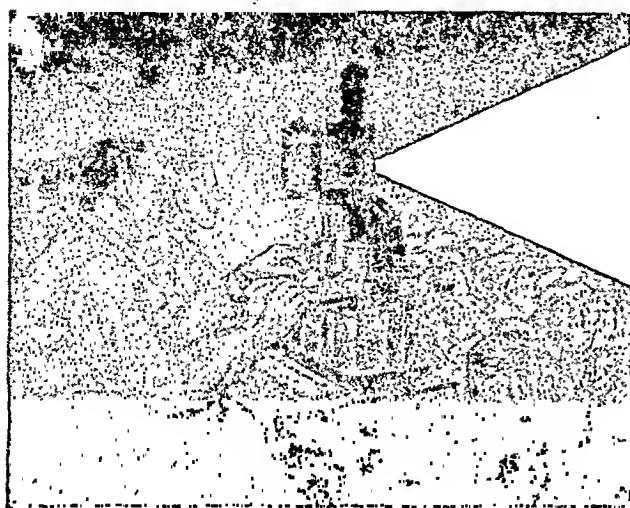
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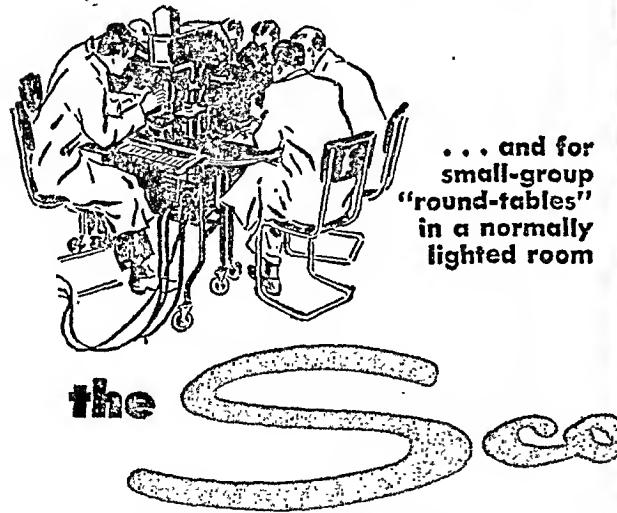
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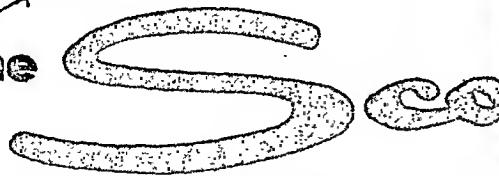
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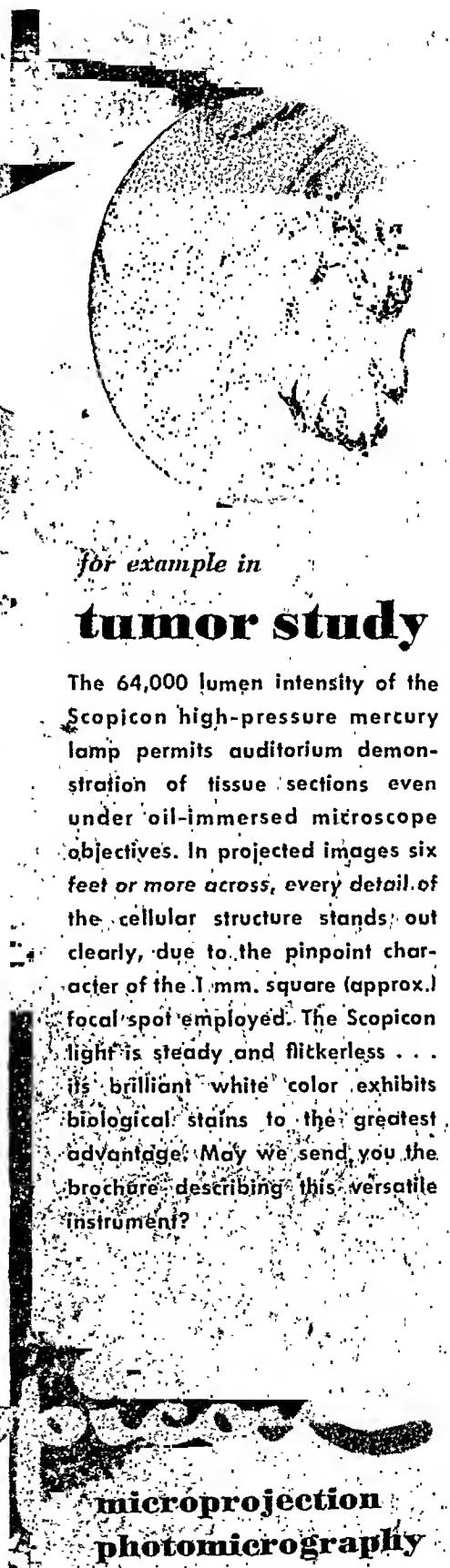
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